Studies on *Theileria parva* in *Rhipicephalus appendiculatus*

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Declaration

I hereby declare that the work presented in this thesis is the product of my own efforts and has not been submitted in any previous application for another degree. The work on which this thesis is based is my own except where stated in the text or acknowledgements.

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ABBREVIATIONS

~	approximately
%	percentage
<	less than
≤	less than, or equal to
>	greater than
≥	greater than, or equal to
=	equal to
^{0}C	degrees centigrade
µg	microgram
µl	microlitre
µM	micromole
µm	micron
ACD	acid citrate dextrose
a.u.	activity units
bp	base pairs
BPA	bovine plasma albumin
BSA	bovine serum albumin
cm	centimetre
CO_2	carbon dioxide
CTVM	Centre for Tropical Veterinary Medicine
DIG	digoxygenin
DNA	deoxyribonucleic acid
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanosine triphosphate
dTTP	deoxy thymidine triphosphate
dUTP	deoxy uracil triphosphate
dsDNA	double stranded DNA
ECf	East Coast fever
EDTA	ethylenediamine tetra-acetic acid
f	female
Fig.	figure
Fl-dCTP	fluorescene-conjugated deoxy cytosine triphosphate
g	gram
x g	relative centrifugal force
GUTS	ground up tick supernatant
hr	hour
H_2O	water
HCl	hydrochloric acid
IFAT	indirect fluorescent antibody test
ILRI	International Livestock Research Institute
ISH	*in situ* hybridisation
Kb	kilobases
kg	kilogram

l	litre
M	molar
m	male
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
MGP	methyl green and pyronin
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	millimole
mm	millimetre
no.	number
n	number of samples
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT/BCIP	4-nitrobluetetrazoliumchloride/5-bromo-4-chloro-3-indolyl-phosphate
ng	nanogram
nm	nanometre
NVRC	National Veterinary Research Centre, Kenya
NZW	New Zealand White [rabbit]
OD	optical density
PBM	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
pg	picogram
PK	Proteinase K
rbc	red blood cell
r.h.	relative humidity
RNA	ribosenucleic acid
RPG	right parotid lymph gland
s.d.	standard deviation
SDS	sodium dodecyl sulphate
spp.	species
SSC	saline-sodium citrate buffer
Taq	*Thermus aquaticus*
TBE	tris-borate-EDTA solution
TBS	tris buffered saline
TE	tris-EDTA
t.e.	tick equivalent
TPR1	*T. parva* repetitive region
u	units
UV	ultra violet
w/v	weight per volume

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ABSTRACT

Aspects of the association between the haemoprotozoan parasite *Theileria parva* and its tick vector *Rhipicephalus appendiculatus* were investigated.

Examination of dissected and stained tick salivary glands by light microscopy is the traditional technique for assessing tick infections. Field collected ticks are often alcohol preserved, or die before they are returned to the laboratory, which precludes dissection. A PCR was developed that detected *T. parva* within alcohol preserved ticks, as this is commonly the state in which ticks are brought back from field collections. Parasite detection by this method was highly correlated with that of microscopy. The technique was then used on cattle and tick samples collected from three field sites in Kenya to assess *T. parva* prevalence. One field site (Limuru) had been vaccinated with a live 'infection and treatment' vaccine, while the other two areas (Kakamega and Kitale) were unvaccinated at that time. Limuru showed lower *T. parva* prevalence in cattle than may have been expected if a carrier-state had been created as a result of vaccination. All the cattle sampled in Kakamega and Kitale were infected with an unidentified *Theileria* species and tick infections ranged from 1.5% to 20% respectively. The results from the three areas were discussed in relation to the effect of the vaccine in Limuru and the implications of vaccine introduction into Kitale and Kakamega.

The distribution of *T. parva* infection in populations of ticks can be characteristically represented by a negative binomial distribution. The dynamics of infection need to be better understood for a number of reasons. Ticks are used as the raw material for the 'infection and treatment vaccine' and the production process could be greatly improved by reliably obtaining a greater abundance of less aggregated tick infections. Another reason is that mathematical models for disease prediction and control would greatly benefit by increased knowledge of parasite dynamics through the tick. *R. appendiculatus* infected with *T. parva* and control, uninfected ticks (for comparison) were dissected at regular times throughout their moult, fixed and

embedded in plastic. Examination of the Giemsa's stained tick sections provided information on the constant decline of parasite numbers throughout the tick moult. The greatest percentage decline was in the gut by excretion and digestive processes. Of the surviving parasites, the mature zygote to kinete forms appeared to suffer the greatest reduction in numbers within the gut digestive cells. They also showed that the number of parasites was highly variable between ticks, throughout every stage of the tick moult.

Inducible immune responses have been shown in a number of haematophagous arthropods. Because ticks are so long-lived, an acquired immune response is likely to be of great benefit to them. Adult infection rates were compared in two batches of ticks, one that had been exposed to *T. parva* as larvae and nymphs and the other just as nymphs. The results showed a significant reduction of infection in the twice infected ticks compared to the once infected controls, suggesting a powerful anti *T. parva* immune mechanism had been triggered as a result of secondary exposure to the parasite.

Disease caused by *T. parva* is usually regarded entirely in the context of the infection in cattle. However, considerable parasite induced pathology was seen in the ticks. This included damaged gut, Malphighian tubules and salivary glands, leading to impaired feeding performance, bloodmeal digestion and tick development throughout the moult. Significantly reduced reproductive capacity was also noted in the infected ticks.

In situ hybridisation was used to detect the pathogen in tick tissue sections. This method was seen as preferable to Giemsa's staining because it could make parasite identification quicker and less subjective. The technique was unsuccessful, probably because of the fixation conditions used. The technique and means by which it could be improved are discussed.

CHAPTER 1

Summary statement of the background and purposes of this study

Only a tiny percentage of the piroplasms ingested by a tick in an infected bloodmeal go on to become infective sporozoites in the salivary gland of the next tick instar. An overdispersed distribution of *Theileria parva* infection is usually seen within *Rhipicephalus appendiculatus* populations, which means the majority of ticks show little or no infection and the majority of the parasites are concentrated within a minority of the ticks. The mechanisms behind these phenomena are poorly understood. The need for improved production of live, parasite vaccine and for more accurate mathematical models of disease are the main driving forces behind research into this area. The overall purpose of this study was to provide more accurate information on the passage of *T. parva* through *R. appendiculatus*. This involved assessing the infection levels in Kenyan field collected ticks using a polymerase chain reaction test (PCR) developed from laboratory based transmission experiments. The focus was then centred on vector competence aspects of the tick-parasite relationship. This included the quantification of different developmental forms of *T. parva* in the ticks and documenting the pathogenic effects of the parasites on their tick vectors.

T. parva is a haemoprotozoan parasite which infects *R. appendiculatus* ticks and cattle in southern, eastern and central Africa. The cattle disease, East Coast fever (ECf) is one of the most economically important restrictions to livestock production in the eleven countries affected. It is calculated that around 25 million cattle are at risk from the disease every year and direct economic losses have been calculated at US$168 million, including an estimated annual mortality of 1.1 million cattle.

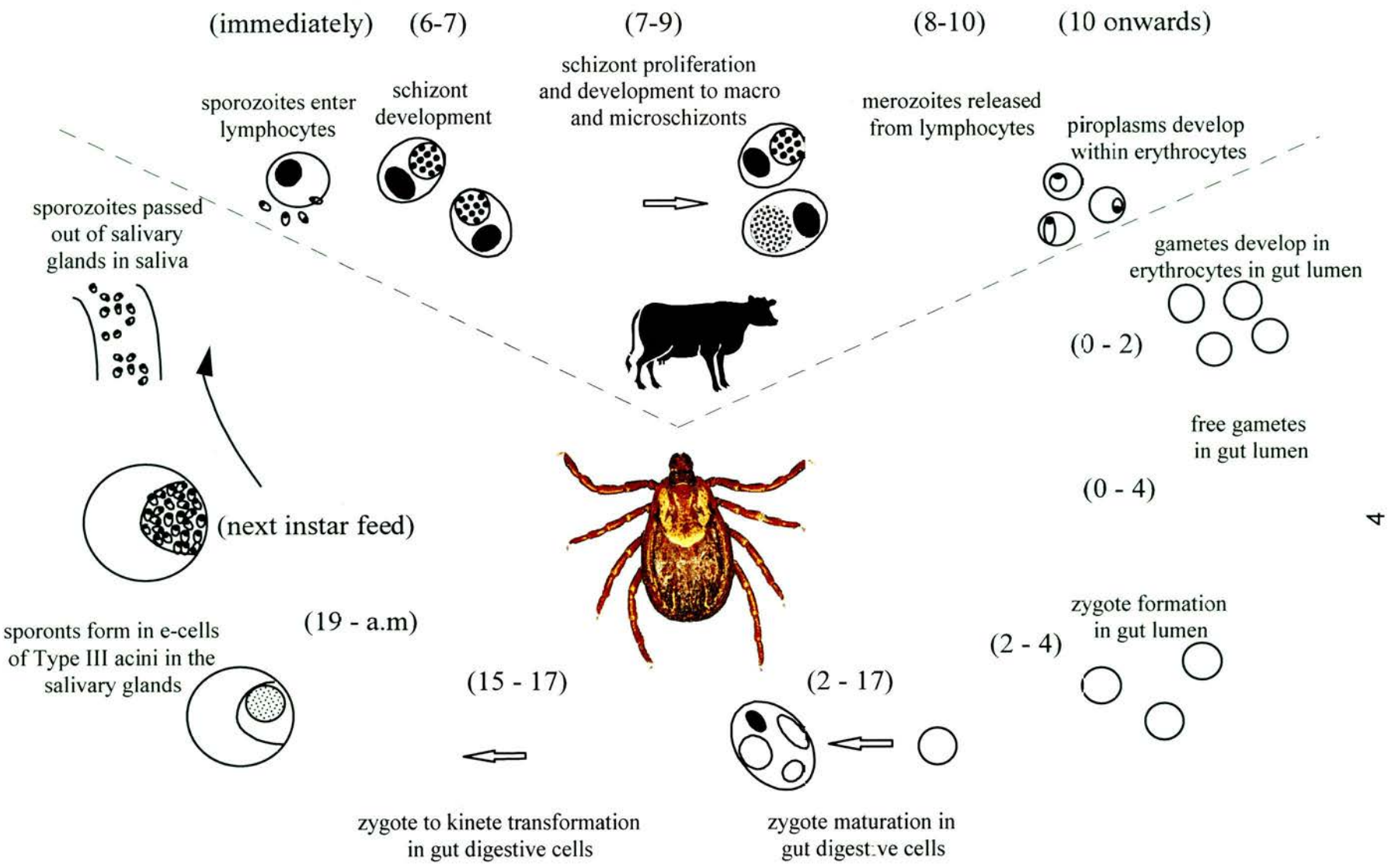
R. appendiculatus is a three-host tick which has a very wide host range. Cattle are the major domestic animal hosts and support all three tick instars. Wild ungulate host species include buffalo, giraffe, eland and waterbuck, and these can become heavily

infested. The tick's geographical range varies depending on climatic conditions, contracting and expanding around its borders, usually in response to variations in precipitation and temperature, with the ticks being far more prevalent in areas with high rainfall. The distribution of theileriosis is closely linked to that of *R. appendiculatus*, but does not occur throughout the entire range of the tick.

European type Taurine cattle and their crosses with native zebu cattle which have been developed to increase productivity are particularly susceptible to the disease and experience very high morbidity and mortality rates. In many areas, farmers are encouraged to keep the less profitable zebu breeds as they are more resistant to ECf and tick infestation. Even with acquired immunity to *T. parva*, zebu calves can still suffer retarded growth.

Fig. 1.1 shows a lifecycle diagram of the parasite. Sporozoites enter the mammalian host in tick saliva and very rapidly penetrate lymphocytes. The infected cells then accumulate in the local drainage lymph node where they undergo multiple cycles of schizogony. The piroplasm is the mammalian intraerythrocytic form, sometimes also referred to as the gametocyte in common with *Plasmodium* nomenclature. When these enter the tick gut, they transform, still within the erythrocytes, to gametes, before the red cells are lysed and the gametes are liberated into the gut lumen. It is at this point that fertilisation occurs between male and female gametes to form a zygote which quickly crosses the gut digestive cell membrane to continue its development in the cytoplasm of the host cell. Over a two-week period, the zygote grows considerably larger and then develops into the motile kinete stage. The kinete migrates from the gut digestive cells and penetrates the salivary glands where it develops into a sporont. When the salivary glands are stimulated, either by tick feeding or heat application, sporogony begins and the sporont becomes a sporoblast. This goes on to mature and form uninucleate sporozoites, which are infective to appropriate mammalian hosts. The sporozoites infect lymphocytes and develop into macroschizonts. Successive rounds of division (schizogony) lead to more

Figure 1.1. Diagram illustrating the life cycle of *T. parva* in cattle and *R. appendiculatus*. The numbers in brackets represent the days (post detachment in the tick) at which each *T. parva* form could expect to be seen in cattle and ticks. The different parasite forms have not been drawn to scale. a.m - adult moult. * - the parasite development times mentioned for the ticks refer to CTVM laboratory infections based on ticks incubated at 28^0C.


(immediately)
(6-7)
(7-9)
(8-10)
(10 onwards)
sporozoites enter lymphocytes
schizont development
schizont proliferation and development to macro and microschizonts
merozoites released from lymphocytes
piroplasms develop within erythrocytes
sporozoites passed out of salivary glands in saliva
gametes develop in erythrocytes in gut lumen
(0 - 2)
free gametes in gut lumen
(0 - 4)
(next instar feed)
(19 - a.m)
sporonts form in e-cells of Type III acini in the salivary glands
(15 - 17)
(2 - 17)
(2 - 4)
zygote formation in gut lumen
zygote to kinete transformation in gut digestive cells
zygote maturation in gut digestive cells

macroschizonts and microschizonts. The parasites then undergo merogony that results in merozoites bursting out of the lymphocytes to infect erythrocytes.

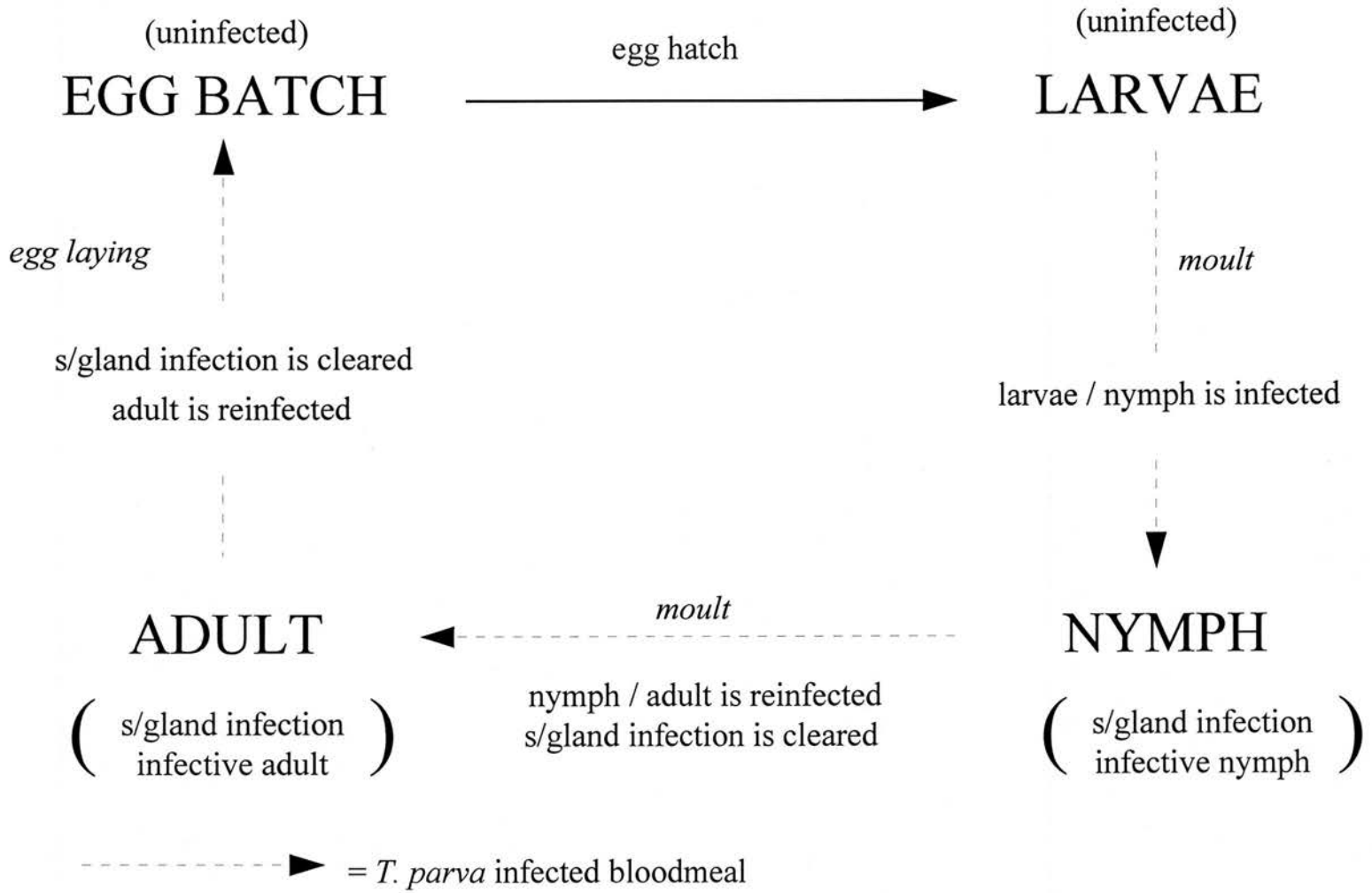
Only the tick associated stages of the *T. parva* lifecycle will be elaborated on in this thesis.

Theileria transmission in the tick is transstadial, which means that infection acquired at one instar from the bloodmeal is transmitted at the next instar. It is widely accepted that transovarial transmission does not occur with *Theileria*, meaning the only instars capable of transmitting the disease are the nymph and adult. Fig. 1.2 shows a diagram representing the times at which the ticks are capable of acquiring and transmitting *T. parva*. If larvae engorge on infected blood, the tick will be **infected** during the larval/nymph moult. The emerged, unfed nymphs may or may not be **infective** by harbouring infection in their salivary glands.

If nymphs feed on infected blood, the existing salivary gland infections will be cleared as a consequence of tick feeding mechanisms, but the ticks will be infected during the nymph/adult moult. The emerged, unfed adults may be infective by harbouring infection in their salivary glands. Because transovarial infection does not occur, adult ticks are capable of transmitting infection they acquired as nymphs, but not of allowing any new infection to develop to an infective stage.

Acaricide application is the most common means of controlling ECf. However, there are a number of problems with this approach, not least of high costs, that are stimulating research into other means of control. Toxic residues in meat and milk with some acaricides mean that sale of animal products have to be restricted for an agreed time after use. There are also environmental concerns about wide spectrum activity against non-parasitic invertebrates that may be affected by chemical run-off. Another major concern is that of resistance. Repeated use places tick species under selection pressure in that ticks which possess some resistance are most likely to

Figure 1.2. Diagram illustrating the *R. appendiculatus* instars capable of acquiring and transmitting *T. parva*. The figures in brackets for the larval and nymph feeds refer to the moulting and post-moult development times of CTVM laboratory ticks incubated at 28^0C.


(uninfected)
EGG BATCH
egg hatch
(uninfected)
LARVAE
egg laying
moult
s/gland infection is cleared
adult is reinfected
larvae / nymph is infected
ADULT
moult
NYMPH
nymph / adult is reinfected
s/gland infection is cleared
(s/gland infection infective adult)
(s/gland infection infective nymph)
= T. parva infected bloodmeal

survive and reproduce and ultimately their progeny predominate. One of the dangers with uncontrolled or badly managed acaricide use is that resistance could become so widespread that it would outstrip the development of new, effective treatments. Very regular, tightly managed dipping regimes have eliminated ECf from large areas, such as South Africa, but this requires a level of infrastructure and management that is not practicably possible in all the areas included within the geographic distribution of *R. appendiculatus*.

Very effective curative drugs are now available for the treatment of ECf and some can be administered as a prophylactic. However, the costs are very high and prohibitive for the majority of farmers.

Vaccine administration is seen as a central component for future disease control. There are two vaccine candidates at present; a recombinant protein and a live parasite vaccine that is administered with a dose of long acting curative drugs. The recombinant protein vaccine - P67 has been developed at the International Livestock Research Institute (ILRI), Kenya. It presently offers limited protection, but future development should significantly improve on this. The live vaccine is most likely to be commercially available in the near future. Ground up tick supernatant (GUTS) stabilate is prepared by partially feeding infected adult ticks, to allow sporozoite formation in their glands. The partially fed ticks are homogenised and diluted in appropriate tissue culture medium, cryoprotectant added and the suspension is aliquoted into vials that are then stored in liquid nitrogen. The immunising dose is injected subcutaneously near a peripheral lymph node and long-acting tetracyclines are given to interfere with the early stages of parasite development. This permits the parasite lifecycle to continue in a limited manner, which allows the animal to develop immunity against the parasite without developing severe clinical symptoms. The development of this type of live vaccine suffers from a number of problems some of which are; batch to batch variation due to varying tick infection outcomes, the development of drug resistance because of parasite variation after recombination in

the tick, the high labour costs involved in the production and delivery process etc. A greater understanding of the pre-sporoblast tick/parasite interactions may allow the system to be manipulated to result in a more standardised infection profile within the tick batches, thereby improving the quality of the vaccine.

One of the most important and fundamental aspects of this type of vaccination from an epidemiological point of view is the creation of a carrier-state within treated cattle. The carrier-state is defined as the ability of an infected and recovered host to infect ticks, which are then able to transmit the parasite to susceptible hosts. If every animal within an area become a carrier, it may have huge implications for tick transmission and parasite dynamics throughout the whole system. During a primary *T. parva* infection, cattle usually maintain relatively high piroplasm levels that are likely to result in high tick infections. However, high piroplasm parasitaemias are generally short-lived. Thus, infections established in ticks as a result of their feeding on carrier animals are likely to be more significant in determining infection rates in the tick population as a whole than infections acquired by ticks feeding on animals undergoing primary infections. Most of the laboratory work carried out to date has involved ticks feeding on cattle with much higher *T. parva* parasitaemias than are likely to occur in the field so that high tick infections could be obtained. It is now becoming clear however, that increased research into the infection dynamics of carrier-state infections is necessary for a better understanding of the ecological aspects of the disease.

The lifecycle of *T. parva* in *R. appendiculatus* involves the parasite surviving in, and penetrating a number of different tissue types. The gut lumen is the first of these, followed by the gut digestive cells, the haemocoel and finally the salivary glands. At each of these critical points, a large reduction in the number of parasites occur, so that the final number of developing sporoblasts in the salivary glands will represent a tiny fraction of the number of piroplasms initially ingested with the bloodmeal. Most of the research on *T. parva* in *R. appendiculatus* has focused on the sporoblast and sporozoite forms and has tended to ignore the gamete, zygote and kinete forms.

Infected ticks are used as the raw material for the live *T. parva* vaccine. An increased knowledge of the parasite/tick relationship will be essential to better understand the factors that influence the infection outcome in the ticks. One of the major phenomena seen in *T. parva* infections of most outbred and selected lines of ticks is the overdispersed infection distribution. This means that the majority of the parasite population will be concentrated in a minority of the tick population. For live vaccine production, the ideal situation would be to have a normal *T. parva* distribution within *R. appendiculatus* as the system would be more manipulable and infection outcomes would be easier to predict.

Mathematical modelling is an essential part of theileriosis research and management because of the multitude of complex interactions encompassed within every disease situation. These include vector abundance and distribution, climatic factors, immunological status of host populations, vegetation type and land use to name but a few. One of the critical factors involved in the equations is the ability of *Theileria* to survive in the vector. Very low prevalence and abundance values are recorded in most surveys of *T. parva* infections in field ticks. Laboratory transmission experiments result in highly overdispersed tick infections and often show non-linear relationships between bovine and subsequent tick infection levels. The underlying mechanisms of these phenomena are as yet unclear, but a greater understanding of the *T. parva*/*R. appendiculatus* relationship will be essential to improve the accuracy of existing mathematical models and to allow the development of more sophisticated control strategies that are able to include the relevant tick infection components into their framework.

Introduction and literature review

The protozoan *Theileria parva* is placed in the Phylum Apicomplexa and belongs to the Class Sporozoea (Levine *et al* 1980). It is highly pathogenic to cattle causing Corridor disease (South Africa), January disease (Zimbabwe) and East Coast fever (ECf) (eastern Africa). The clinical disease caused by *T. parva* differs depending on geographic region, but is always associated with the proliferative and destructive phases in the lymphocytes. The economic cost of the disease has been estimated by Mukhebi *et al* (1992) who calculated that the greatest losses were seen in the following areas; milk production, acaricide purchase, loss of draught power and beef production, in that order. The authors stressed the results were based on inadequate data and that ongoing research should lead to the development of better models.

There are a number of closely related theilerial species that also occur in the African continent. *Theileria annulata* infects cattle and the Asiatic buffalo (*Bubalus bubalis*), and like *T. parva* can be highly pathogenic in Taurine cattle, causing Mediterranean or tropical theileriosis. The disease is transmitted by *Hyalomma* ticks and is confined mainly to the countries north of the Sahara desert. Although the schizonts are indistinguishable from those of *T. parva*, misdiagnoses are rare because of the geographic separation of the two disease ranges. *Theileria mutans* and *Theileria velifera* are also parasites of African buffalo and cattle and often occur in the same areas as *T. parva*. It is entirely possible for cattle in these areas to have mixed infetions. Both parasites are usually benign in cattle, are transmitted by *Amblyomma* ticks, and are very difficut to distinguish from *T. parva*. *Theileria taurotragi* is principally a parasite of eland but has also been shown to infect cattle (Young *et al* 1977). It is transmitted by *R. appendiculatus* and is morphologically indistinguishable from *T. parva* in the tick salivary glands. Discriminating *T. taurotragi* from *T. parva* is very difficult in cattle and ticks by microscopy, but can be done at a molecular level (Allsopp *et al* 1993, Bishop *et al* 1994). A number of other tick-borne diseases can be transmitted by ticks capable of transmitting *Theileria*. The most economically important of these is heartwater and involves the rickettsia *Cowdria ruminantium* being transmitted by *Amblyomma* ticks. The disease

is manifest by pyrexia (≥41^{0}C), nervous and respiratory symptoms and death occurs in peracute cases within 36 hours. All domestic and wild ruminants are susceptible to the disease, but only the former seem to be really sensitive while the latter act as reservoirs.

Indigenous cattle such as the East African Short Horned Zebu (*Bos indicus*) are much more resistant to theileriosis and tick infestation than more productive European breeds such as the Friesian (*Bos taurus*). Moll *et al* (1984, 1986) showed that low numbers of zebu calves die in endemically stable areas whereas the mortality rate in taurine cattle is very high unless strict control measures are adopted. In many areas, farmers are restricted from keeping improved breeds because of the threat posed by the disease (Callow 1983), thereby greatly inhibiting the economic growth of these areas.

The distribution of theileriosis is closely associated with that of *R. appendiculatus*. The tick's distribution is affected by a number of factors, the most important of which are climatic factors and vegetation (Branagan 1973). The climate matching model CLIMEX (Sutherst and Maywald 1985) was designed to estimate the distribution of arthropod pests and vectors in relation to metereological conditions. Perry *et al* (1990) applied CLIMEX to the interpolated climate database in Africa for predicting areas of *R. appendiculatus* distribution. The predictive index was very closely correlated with recorded tick distribution. Geographic and social factors such as dense forest to the west, low cattle stocking and minimal cattle movement in Zaire and arid regions in northern Kenya have served to limit the ticks' range (Perry *et al* 1990). The distribution of *R. appendiculatus* is constantly advancing and retreating around its borders in response to wet and dry climatic cycles (Norval and Perry 1990). There are great climatic differences over this area, with seasonality being much more apparent with increased distance from the equator. The daily mean temperature in Burundi varies by 2 to 3°C per year, whereas in the Cape Province of South Africa, this variation may be as much as 12 to 15°C which has significant impact on tick development rates, in that they moult faster at higher temperatures

(Randolph 1997). As a consequence of the variable climates in Southern Africa, the ticks show a diapausing response at certain times of the year that means they cease questing and become quiescent, a behavioural adaptation thought to be controlled by varying day lengths (Berkvens *et al* 1995). Transmission of ECf will be greatly influenced by the differences in tick behaviour, making a fuller understanding of the dynamics and mechanisms of *T. parva* development within *R. appendiculatus* at different temperatures of critical importance to epidemiology and vaccination programmes.

East Coast fever does not occur throughout the entire *R. appendiculatus* range as *T. parva* development within the ticks is affected by factors such as temperature. Cowdry and Ham (1932) were the first to demonstrate the effect of temperature on the development of *T. parva* in *R. appendiculatus*. Lewis and Fotheringham (1941) and Lewis (1950) were less interested in the dynamics of the parasite within the tick and more in the resulting sporoblast infections and the clinical reactions these produced in cattle. Their work showed a linear relationship between incubation temperature and tick moulting times and the eventual tick infections. Young and Leitch (1981a) confirmed and extended the earlier work, particularly regarding the parasite developmental stages throughout the tick moult, rather than just the sporoblast infections in the salivary glands. Incubation temperatures of 23^0C and 28^0C produced the highest levels of infection depending on the parasite stock used, indicating the importance parasite genotype has on the outcome of infection. Young and Leitch (1981b) showed that varying daily temperature between 25^0C and 7^0C produced significantly higher infections than at a constant temperature, presumably because it was more representative of field conditions. Separating the effects of temperature and tick moult on parasite development is not possible as *T. parva* development is directly controlled by the moult, which is in turn directly controlled by temperature (Young and Leitch 1980). Motile kinetes appear in the gut epithelial cells one day prior to moulting in larvae/nymphs (day 9 at 28^0C) and nymph/adults (days 13-14 at 28^0C) or nymph/adults (day 28 at 23^0C), showing there is not a single predetermined time limit for their development (Young and Leitch 1981a). They

suggest that *T. parva* does not develop in *R. appendiculatus* at temperatures below 18^0C and above 37^0C because the parasite is unable to co-ordinate its development to the extremes of tick moulting times that result. The same temperature related effects with regard to moulting times and parasite development have been noted in *Hyalomma annatolicum* ticks infected with *T. annulata* (Das and Sharma 1991).

Carrier-state and endemic stability

Norval *et al* (1992) provide an excellent review of the carrier-state in cattle and describes how the lifecycle is maintained by the schizont and piroplasm stages, both of which are thought to show very slow proliferation to evade hostile immune responses. The carrier-state appears to be more highly developed in buffalo, which can remain infected for considerably longer than cattle and is a very important component of *T. parva* epidemiology in areas where cattle are in close contact with wildlife. A naturally infected buffalo was shown to be a carrier after 12 years of tick isolation, and was still able to infect *R. appendiculatus* ticks at higher prevalence and abundance values than carrier cattle (Young and Grootenhuis 1985). A *T. parva lawrencei* experimental infection showed another buffalo to be a carrier up to day 888 post-infection (Grootenhuis *et al* 1987).

Short and Norval (1981) considered the carrier-state essential for infecting *R. appendiculatus* nymphs with *T. parva bovis* based on disease occurrence in relation to the feeding times of the tick instars. Later work showed that cattle that were kept tick free remained carriers for 7 to 9 months post infection (Koch *et al* 1992).

Young *et al* (1981) demonstrated a *T. parva* carrier-state in cattle from an endemic area of Kenya. Five isolated animals remained positive for *T. parva*, *T. taurotragi* and *T. mutans* antibodies throughout the course of the 9 month experiment and were shown to be *T. parva* and *T. velifera* carriers over the same period by their ability to infect ticks. Kariuki *et al* (1995) immunised cattle with *T. parva* sporozoite stabilate and later demonstrated the carrier-state using infection results from ticks and cell culture. *R. appendiculatus* nymphs were placed on the animals 3 and 7 months after

immunisation and the tick salivary glands were examined for infection after they had moulted to adults. Blood samples were taken from the cattle on both occasions and *T. parva* infected cultures were set up from the separated lymphocytes. This was the first demonstration of piroplasms and schizonts in carrier cattle.

Minjauw *et al* (1998) carried out a vaccination trial in Zambia in which they had four groups of cattle, vaccinated with tick control, vaccinated without tick control and unvaccinated with tick control and unvaccinated without tick control. The results of an artificial challenge experiment at the end of the field trial showed that the *T. parva* vaccinated cattle displayed no, or very mild clinical reaction to sporozoite stabilate challenge, whereas most of the unvaccinated, control cattle showed very severe clinical reactions. ECf incidence in the non-vaccinated experimental cattle, kept in the same area as the vaccinated cattle markedly declined six months after the experimental cattle in the same area were immunised. This suggests that the carrier-state induced by immunisation did not lead to a persistent high incidence of ECf, even in unvaccinated cattle and might accelerate the development of endemicity.

Endemic stability to ECf has been defined by Norval *et al* (1992) as the state in a cattle population where the large majority of the population becomes infected and immune by 6 months of age, and little or no clinical disease occurs. Endemic instability describes a state in which only a small proportion of the cattle in a population becomes infected and immune by 6 months of age, and clinical disease is seen. Native zebu cattle are susceptible to ECf when endemic stability has been broken, either by being on the edge of *R. appendiculatus* distribution or by dipping and other factors which reduce challenge. Deem *et al* (1996) gave three requirements for endemic stability to be attained in tick borne diseases; a high prevalence of infection in vector ticks, a large reservoir of infection in vertebrate hosts and that vertebrate hosts become infected even when resistance to clinical disease is high.

Endemic stability has been found with a number of tick-borne diseases, such as heartwater. Jongejan *et al* (1988) demonstrated *Amblyomma* ticks could become

infected with *C. ruminantium* by apparently healthy, indigenous cattle from an endemic area. The reason it took so long to establish the idea that a *C. ruminantium* carrier-state existed was because larvae had always been applied to infected hosts and infection assessed in the moulted nymphs, which tend to have very low prevalence and abundance of infection. However, Andrew and Norval (1989) showed high infection of adult ticks which had fed as nymphs on a number of *C. ruminantium* infected and recovered ruminants. Field studies of *Amblyomma hebraeum* collected from an endemic area in Zimbabwe showed very high *C. ruminantium* infection prevalence of up to 36% in female and 45% in male ticks (Norval *et al* 1990). Heartwater is somewhat different from other tick-borne diseases in that transplacental transmission can occur and this has a significant impact on the dynamics of endemic stability (Deem *et al* 1996). Eriks *et al* (1993) stated that persistently infected cattle with low rickettsaemias were more important in the epidemiology, and consequent endemic stability of anaplasmosis than acutely infected animals displaying high rickettsaemias.

The infection of all young calves throughout their first disease season was reported to be critical for the maintenance of endemic stability to *Babesia bovis* (Mahoney 1977). In many low-income areas, the maintenance of endemic stability is essential as the costs of drugs and/or intensive tick control is prohibitive. Tice *et al* (1998) recommended strategic dipping to reduce tick numbers but not eliminate them, so that continual disease challenge/boosting could occur. In an attempt to integrate the same theory with an extremely low cost approach, Dreyer *et al* (1998) investigated the efficacy of applying used engine oil onto feeding ticks as a control measure. Their results showed that tick numbers were reduced (although less than if acaricide had been used) and that endemic stability to *A. marginale* and *B. bigemina* was unaffected.

Intimate knowledge of the endemic stability of any disease situation is essential before vaccination can be considered for an area. Taurine cattle, which are highly susceptible to ECf, need to be protected by either vaccination or tick control

strategies. Zebu cattle are usually resistant to clinical disease caused by *T. parva*, but in situations where endemic normality breaks down, which can happen for a variety of reasons, vaccination may be considered (Deem *et al* 1993).

Vaccination

Theileria annulata is very successfully controlled by a live, cell culture vaccine. The vaccine is used in many *T. annulata* endemic areas from Morocco to China (Brown 1990). Virulence of *T. annulata* schizonts can be attenuated by prolonged growth in cell culture (Pipano 1981). After the cells have been grown for a considerable period of time in culture, schizonts inoculated into susceptible animals stop producing clinical reactions typical of ECf (Pipano *et al* 1973). Neither schizonts or piroplasms can be found in the vaccinated animals. Bulk cultures can be produced and cryopreserved very efficiently. It was anticipated that a similar approach could be used for *T. parva* vaccination. However, this was not possible as susceptible animals only became reliably infected when inoculated with schizont infected, autologous cells (cultures which derived from their own peripheral blood mononuclear cells) (PBM) and not allogeneic cells (cultures which derived from cells of other cattle) (Morrison *et al* 1981). Dolan *et al* (1984a) demonstrated a histocompatibility barrier for cattle that were inoculated with cells that did not match their bovine lymphocyte antigen (BOLA) profile. This meant an immune reaction was generated against the inoculated lymphocytes themselves, destroying them and the schizonts within them. Morrison *et al* (1981) showed that cattle could be infected with as few as 10^2 *T. parva* infected, autologous cells whereas 10^8 allogeneic cells were required to induce infection. *T. annulata* does not show a histocompatibility barrier and so heterologous cells can be used for vaccination. The reasons why *T. parva* and *T. annulata* differ in this regard have not been clearly established, but may be involved with the different bovine cells the two parasites infect. *T. parva* infects T cells whereas *T. annulata* infect macrophages (R. Spooner; CTVM, personal communication).

Tick infection followed by treatment with long acting antibiotics such as tetracycline produces solid immunity in immunised cattle (Neitz 1957, Brocklesby and Bailey

1962, Radley *et al* 1975) and was referred to as 'infection and treatment'. The initial method of Neitz (1957) used application of laboratory infected ticks to cattle followed by drug treatment. However, cattle infections were unreliable and often fatal because the tick infections were difficult to predict, meaning the challenge dose tended to vary considerably. The ability to produce and store sporozoite stabilates (Cunningham *et al* 1973) was a significant breakthrough for this approach in that it allowed standard quantified doses of sporozoites and a reduction in the amount of tetracycline necessary after stabilate inoculation (Brown *et al* 1977). It is important to select vaccinating strains that protect against a wide spectrum of *T. parva* strains as certain cattle and buffalo derived *Theileria* stocks have broken the chemoprophylactic cover afforded after infection and treatment vaccination. Radley (1981) and Mutugi *et al* (1990) have shown that most of these breakthroughs can be controlled effectively by increasing the period of oxytetracycline cover.

The sporozoite vaccine is prepared by grinding up *T. parva* infected, partially fed *R. appendiculatus* (to allow sporozoite development to occur) in suitable culture medium. The mixture is then centrifuged and the sporozoites, contained within the supernatant are removed. More medium and the gradual addition of cryoprotectant to the sporozoite suspension precedes aliquoting and storage in liquid nitrogen. For use in vaccination, the aliquots are removed from storage, rapidly defrosted and equilibriated at room temperature (~25^{0}C) for 15 to 40 min and then subcutaneously injected near a draining lymph node; usually around the head or shoulders. Drug treatment is given at the time of parasite inoculation and the concentration and number of doses depend on the local *Theileria* field strains. The infection dynamics of the *Theileria* stocks used will have been established beforehand in experimental cattle. Use of the vaccine has a number of drawbacks. Acaricide application is still necessary because of the risk of other tick-borne diseases, although the quantities applied can be considerably reduced. Live parasite vaccines can pose considerable safety drawbacks for large-scale immunisation programmes. Costly, extended monitoring is required and the liquid nitrogen cold chain is a considerable technical challenge for vaccination in many remote areas. Broad-based protection against the

widest range of different species, strains and antigenic types of *Theileria* is one of the most important criteria used in selecting the vaccine candidate stocks. The ''Muguga Cocktail'' was composed of three stocks; *T. parva* Muguga, *T. parva* Kiambu 5 and buffalo-derived *T. parva* Serengeti transformed (Radley *et al* 1975) and found to give good protection in the laboratory and field against cattle derived *T. parva* (Radley 1981, Muisi 1990), but poor protection against buffalo derived parasites (Radley *et al* 1979). More recently, *T. parva* Marikebuni (isolated from the Kenyan Coast Province) has been found to offer excellent protection against a wider range of cattle derived *T. parva* (Mutugi *et al* 1990). Some countries have been unwilling to vaccinate with *T. parva* stocks from outwith their area because vaccinated cattle, which are likely to become carriers, may introduce new stocks of parasite into their country. However, there are indications that considerable homogeneity does exist between *T. parva* stocks in Africa as *T. parva* Boleni from Zimbabwe protects cattle against a wide range of Kenyan stocks (Irvin *et al* 1989).

Tetracycline is a classic broad-spectrum antibiotic capable of preventing the onset of clinical symptoms associated with *T. parva* (Neitz, 1953, Brown *et al* 1977) and *T. annulata* (Mallick *et al* 1987) infection. It works by suppressing the early development of *Theileria* stages, thereby encouraging host immunity against the parasites, as opposed to being theileriacidal (Norval *et al* 1992). The drug is highly bacteriacidal because it is actively concentrated by prokaryotic, but not eukaryotic cells. It has been shown to inhibit *Plasmodium falciparum* ribosome function, thereby preventing protein production (Pestka 1971). The naphthoquinone derivative, parvaquone was shown to be very effective for the treatment of *T. parva* clinical infections (McHardy *et al* 1979) and Dolan *et al* (1984b) showed this to be true of tick and sporozoite stabilate induced infections. It is thought to inhibit the electron transport within cells (McHardy *et al* 1976) and evidence from electron microscopy shows it kills schizonts and piroplasms without damaging their respective host cells (McHardy 1984, McHardy and Hudson 1984). More recently, another naphthoquinone derivative, buparvaquone, was found to be 20 times more effective against *T. parva* than parvaquone *in vitro* and 8 times more effective *in vivo*

(McHardy *et al* 1985). A significant difference between the two drugs is that cattle treated with parvaquone can become carriers (Dolan 1986), but buparvaquone appears to reduce the development of the carrier-state (Mutugi *et al* 1988).

Another vaccine approach that has been adopted to control tick-borne diseases is against the ticks themselves as opposed to the pathogens they transmit. Johnston *et al* (1986) and Kemp *et al* (1986) inoculated cattle with a crude, tick homogenate and obtained very encouraging results. Some *B. taurus* cattle became almost totally resistant to further tick infestation. Sixty percent of the female ticks that did feed had damaged guts, and greatly reduced fecundity was seen in the others. The damaged guts that were seen mainly in adult ticks (larvae appeared unaffected) caused the bloodmeal to leak into the haemolymph turning the whole tick red. *In vitro* evidence from the studies of Kemp *et al* (1986) suggested that vaccination led to the production of antibodies against gut cell antigens and that antibody, in the presence of complement, resulted in the gut wall damage.

Host resistance

Host resistance to ticks is a very important factor in the epidemiology of tick-borne diseases and may become an increasingly significant factor in future control strategies. Latif *et al* (1991a) studied *R. appendiculatus* resistance in Kenyan zebu cattle and showed that highly tick resistant animals, in comparison to cattle with low resistance, allowed less tick attachment, lower percentages of feeding ticks to engorge fully and lower tick survival rates. They found an overdispersed tick distribution with 12% of the cattle that showed low tick resistance feeding 73% of the ticks. A similar study that recorded numbers of *A. variegatum, Boophilus decoloratus, R. evertsi and R. appendiculatus* on zebu cattle concluded that over the period of the study, highly resistant cattle showed little or no seasonal fluctuations in tick numbers compared with animals of low resistance, which showed an up to sevenfold increase in the magnitude of the tick burden when the tick challenge was high (Latif *et al* 1991b). Cattle with low resistance were responsible for much of the tick multiplication in the field whereas highly resistant animals tended to limit the

populations. In a review of a long-term cattle tick control strategies, Frisch (1999) commented ''Had the effort that has been directed at the development of acaricides over the last 100 years been directed instead towards the development of [cattle] breeds with extremely high tick resistance, it is doubtful there would now be any significant need for acaricides''. The importance of tick resistance in relation to disease transmission was demonstrated by Wikel *et al* (1997). They found that BALB/C mice, which had been repeatedly infested with pathogen free *Ixodes scapularis* nymphs were resistant to infection with tick transmitted *Borrelia burgdorferi*. Tick-naive hosts produce higher *T. parva* infection rates in *R. appendiculatus* ticks than tick-resistant hosts (Fivaz *et al* 1989). Rubaire-Akiiki (1990) showed that *Hyalomma* ticks picked up significantly less *T. annulata* infection from a calf made highly resistant to *Hyalomma* ticks by repeated infestation than from a *T. annulata* infected, tick-naive calf. He commented that this effect, in combination with reduced tick survival after feeding on resistant hosts should have a significant effect on the epidemiology of theileriosis in areas where cattle have high tick burdens.

Grooming behaviour in large ruminants is a very important factor in controlling their tick burdens and in many cases, irritation from the tick feeding site causes great upregulation of the grooming response (Mooring *et al* 1996). Cellular infiltration to tick feeding sites also accompanies host resistance and Riek (1962) showed that larvae can actually be drowned on tick feeding site exudate, limiting numbers even when cattle are prevented from grooming.

Parasite detection methods in ticks

Conventional staining techniques

Koch (1906) and Gonder (1911) were the first to describe *T. parva* development in *R. appendiculatus* by examining Giemsa stained smears with light microscopy. The same technique was used to provide further details by a number of researchers, the most comprehensive of which was by Schein *et al* (1977). Subsequent to this, Mehlhorn *et al* (1978) performed electron microscope (EM) studies on infected ticks

and provided greater morphological detail on the various parasite stages. EM images had previously been provided on *Babesia bigemina* in *Boophilus microplus* by Riek (1964) and these results were later extended by Potgieter *et al* (1976, 1977).

The vast majority of research on *T. parva* in *R. appendiculatus* has focused on the salivary gland stages. Consequently, a number of techniques have been examined to determine the best method for detection. Blewett and Branagan (1973) developed a DNA staining technique using the Feulgen reaction, originally described by Feulgen and Rossenbeck (1924), to be used on slide mounted, whole salivary glands. This made infection detection substantially quicker and quantifiable than the standard method previous to this, which involved examining Giemsa stained sections of infected glands. An alternative method for staining whole glands was reported by Blewett and Branagan (1973) which used methyl green (a DNA stain) and pyronin (an RNA stain) (MGP). This method is particularly effective in detecting sporoblasts, as the hypertrophied host cell nucleus is clearly differentiated as well as the parasite induced, massive RNA synthesis. Another significant benefit of this method over the Feulgen technique, for example, is that pyronin displays colour differentiation in relation to the maturity of the parasite mass; young sporoblasts are bright red whereas those that are mature and ready to transform into sporozoites are purple to blue/black. Voigt *et al* (1995) reviewed ten different staining techniques, concluding that MGP was unsuitable for detecting sporonts and immature sporoblasts and that azure stain, which they considered superior in this regard was best for assessing salivary gland infections in field ticks.

Antibody-based detection methods

The immunofluorescence antibody test (IFAT) has been used to detect *T. parva* in *R. appendiculatus* salivary glands (Kimber *et al* 1973). The technique was found to work well on histological sections but not for whole mounted glands and has not been seriously pursued as an efficient means of parasite detection.

Polymerase Chain Reaction (PCR) - based detection techniques

PCR has almost become a ubiquitous technique in many aspects of biological research (Saiki *et al* 1988) and this is reflected in the use of the technique for pathogen detection in ticks. PCR has a number of advantages over more traditional techniques. A high degree of specificity is possible which can even permit differentiation between closely related strains of the same species. The sensitivity is often greater than other techniques such as microscopical examination of stained preparations or DNA probes. It can be used to detect parasites within archival specimens that would be inaccessible to most other techniques, and utilised for asessing non-fatal biopsy samples. The disadvantages are that it is expensive and prone to false positives as a result of DNA contamination. Davidson *et al* (1999) demonstrated PCR detection of *Borrelia burgdorferi* from ticks. Tiny amounts of haemolymph were removed from the ticks, and if *Borrelia* was detected by PCR, culture was established from the rest of the tick because an increased number of different culture isolates are required to improve diagnostic serological tests. PCR was shown to be a more sensitive method of detecting *C. ruminantium* in *Amblyomma* ticks than the previously available DNA probe (Peter *et al* 1995). *Anaplasma marginale* detection in the haemolymph of *Dermacentor* ticks ensured that only infected ticks would be used in later experiments by Stich *et al* (1993a). The same group then went on to demonstrate the pathogen in salivary secretions from fed and unfed ticks (Stich *et al* 1993b). It would have been difficult to detect these very small parasites in the tiny volumes of haemolymph removed from the ticks by methods other than the PCR.

Kirvar *et al* (1998) used a very sensitive semi-quantitative PCR to detect *T. lestoquardi* infection in *Hyalomma* tick salivary glands. D' Oliveira *et al* (1997) reported a very sensitive PCR that could detect *T. annulata* in alcohol preserved ticks. It was capable of detecting 0.1ng of parasite DNA (corresponding to 12 sporozoites) which had been spiked into DNA extracted from an uninfected *Hyalomma* tick. Another advantage of the PCR over traditional detection techniques was demonstrated by Kawazu *et al* (1995) who used PCR, followed by restriction

enzyme digests to distinguish between *T. sergenti* and *T. buffeli* during mixed infections in their *Hyalomma* vectors. Ohta *et al* (1995) used the PCR to detect very low levels of *Babesia* infection in the eggs and haemolymph of *B. microplus* ticks.

The *Theileria parva* repetitive (TPR1) region of the genome was present in every strain and isolate tested and first referred to by Allsopp *et al* (1989) in relation to its value in strain characterisation. It is a repetitive element of about 1.44 Kb, with about a hundred copies per genome and is transcribed in the piroplasm, but not the schizont stage (Baylis *et al* 1991). *T. parva* DNA from cattle and ticks was amplified by primers hybridising to the TPR1 sequence (Bishop *et al* 1992). The reaction was shown to be more sensitive than light microscopic examination of thin blood smears in detecting parasites in carrier-state cattle. It also detected low level infections in tick salivary glands (as judged by microscopical examination of the paired gland) and schizonts from infected lymph node biopsies.

In-situ nucleic acid detection techniques

In-situ nucleic acid detection techniques can provide an extra layer of information compared to solution reactions by allowing parasite localisation within host tissue. *In-situ* PCR techniques are regularly used to detect viruses in tissue sections (O'Leary *et al* 1997, Marshall and Cubie 1997). Detection of eukaryotic parasites using this technique is relatively uncommon, although it has been reported for *Trypanosoma cruzi* in murine tissue (Lane *et al* 1997). Viruses are more difficult to detect in tissue sections than most parasites making highly sensitive detection techniques like *in-situ* PCR invaluable. The technique is often difficult to establish in tissue (O'Leary *et al* 1996) which may have encouraged parasitologists to investigate other detection methods.

In-situ hybridisation (ISH) has been used to detect the rodent malaria parasite *Plasmodium berghei* in the midguts of *Anopheles stephensii* mosquitoes (Thompson *et al* 1999) and has also been used to detect pathogens in ticks. Booth *et al* (1991) used the method to detect Dugbe virus in *Amblyomma variegatum* and found it to be

most sensitive for viral detection in combination with immunohistochemistry. *Anaplasma marginale* was detected in *Dermacentor andersoni* ticks by ISH, and although detection was very clear in most of the tick body compartments, the rickettsial colonies were more easily detected by conventional light and electron microscopy techniques in the fat body cells (Nie-Lin *et al* 1996).

A number of papers have compared different histological, antibody and nucleic acid based approaches for detection of target in sample tissue (Komminoth *et al* 1990, Bates *et al* 1997, Mulder *et al* 1997). No single system is universally accepted as being better than another. The most appropriate technique very much depends on the nature of the particular tissue under investigation, fixation conditions and a multitude of other factors. The best processing conditions for one type of reaction may be detrimental for another, which means the technique chosen is often that which the researcher gets to work satisfactorily and provides adequately sensitive and reliable results.

Natural tick infection levels

There is a great deal of literature available on assessing parasite infection levels in field collected ticks. The most pertinent papers to this thesis are those on *T. parva* infection in *R. appendiculatus* by Walker *et al* (1981) and *T. annulata* infection in *Hyalomma anatolicum* (Walker *et al* 1983). Examination of MGP stained salivary glands revealed that 1.5% of adult *R. appendiculatus* collected from different Kenyan field sites were infected, with abundance values ranging from less than 1 to 12 sporoblasts per tick. The prevalence of *T. annulata* infection in *Hyalomma* ticks collected from two Sudanese field sites was very considerably higher, ranging from 35 to 91% and with greater abundance values which ranged from 18 to 46. As many as 600 sporoblasts were recorded from individual *Hyalomma* ticks which is extremely high for a field infection.

Mathematical models of disease

A number of mathematical models have been produced to explain and/or predict the incidence of tick-borne disease in a number of areas. They will become increasingly important in evaluating the likelihood of success and the cost effectiveness of future tick-borne disease control programs. There are a large number of factors that are considered in these models that use results from experimental and field based observations. Randolph (1995) stressed the importance of obtaining data from natural host/parasite systems as she found that models based on data from tick transmitted *Babesia microti* were much more realistic than those based on syringe passaged parasites. The infection dynamics of *C. ruminantium* were investigated by O'Callaghan *et al* (1998) using modelling techniques and they showed quantitative and theoretical support for the concept of endemic stability. Trans-placental transmission of the heartwater agent played a major role in the epidemiology of the disease and in that regard made it less comparable with other tick borne diseases. A model assessing the transmission dynamics of ECf was formulated by Medley *et al* (1993). They confirmed that in an endemically stable area carrier cattle are responsible for most *T. parva* transmission, that tick numbers have to be reduced by over 80% to make a significant impact on disease transmission and that using a *T. parva* vaccine in an endemically stable area will have minimum effect. The models generated were not accurate enough for field use, but as the amount of data which can be usefully applied to them increases, so will their predictive and analytical power.

Artificial feeding techniques

There are a number of reasons which make artificial feeding systems attractive to researchers including the cost of laboratory animals, the constant pressure to reduce the use of animals in experimentation and that it can make the study of vector/parasite relationships considerably easier as the parasite density in the bloodmeal can be closely regulated. The technique is particularly convenient and useful for parasite systems where many lifecycle stages can be produced *in vitro*, as

is the case with a number of *Plasmodium* species (Ponndurai *et al* 1982, Janse *et al* 1985, Syafruddin *et al* 1992).

Parasite transmission to a number of haematophagous insects has been carried out using membrane feeding apparatus. Mihok *et al* (1994) artificially fed and successfully infected tsetse flies with trypanosomes and developed an optimum technique for propagating field isolates, which can become less heterogeneous on passage through laboratory hosts. Survival and fecundity of tsetse flies fed on membranes were similar to those fed on rabbits, although the mean pupal weight was reduced in membrane fed lies (Moloo *et al* 1999). *Trypanosoma vivax* infection levels were also found to be lower when the flies were membrane fed.

Sandflies can be successfully fed through chick skin membranes and *Leishmania* transmission has been demonstrated using this system. Hanafi *et al* (1998) showed the feeding characteristics of three different sandfly species were the same on BALB/c mice and membranes and that the level of membrane derived *Leishmania* infections were the same in both systems. The infection outcome of membrane feeding *Lutzomyia* sandflies with *in vitro* and *in vivo* derived amastigotes was investigated by Ismaeel *et al* (1998) who concluded that they were the same, thereby supporting the development of *in vitro* parasite production methods. Sandflies fed on mixed *Leishmania* species showed greater mortality and reduced egg batch weights than those that ingested single species infections (Elsawaf *et al* 1994). This kind of experiment would have been extremely difficult to conduct without access to a membrane feeding system.

Screening the effect of compounds that affect parasite development within a vector is one of the major advantages of a membrane feeding system. Shahabuddin *et al* (1995) added various compounds to infected blood in an attempt to block *Plasmodium* enzymes that are thought to be important for mosquito transmission. They found a correlation between *in vitro* inhibition of a *Plasmodium* trypsin-like enzyme and the reduced level of mosquito transmission. A different approach to

screening for malaria vaccine candidates using membrane fed mosquitoes was used by Toure *et al* (1998). They added human serum (control and from vaccinated humans) to infected blood and fed mosquitoes on this. They were hoping to find that vaccinated, immune serum would inhibit *Plasmodium* development within the vector. However, they stressed that there are many factors other than parasite density in the bloodmeal that account for eventual mosquito infection levels. Membrane feeding assays tend to negate the host-derived differences such as cytokine and antibody levels which contribute greatly to infection outcome. Care should be taken when interpreting the initial results from vaccine trials that have used membrane fed mosquitoes, as they may be unrepresentative of equivalent *in vivo* infection scenarios. This shows that membrane feeding does not replace natural host feeds, but is a useful complement as long as artificial feeding systems and the results from them are used in an appropriate context.

The feeding characteristics of ticks are very complex which makes membrane feeding for acarids more difficult than for other haematophagous arthropods. The length of instar feeds varies depending on species, but usually varies from a few days to weeks during which time an engorged female can increase her bodyweight by over 200 times (Kemp *et al* 1982). There are a great number of elaborate behavioural mechanisms involved in the feeding process which rely on a variety of chemical and physiological stimuli from the host (Waladde and Rice 1982). Early attempts to infect ticks by these means usually involved removing them from a host and placing them on a membrane system for the final engorgement period (Howarth and Hokama 1983). Obviously, this system is not ideal, particularly for female ticks as it is physiologically disruptive for them to be forcibly detached during the feeding process, although adult ticks were able to transmit *A. marginale* when infected as nymphs through a membrane.

There have been a number of successful parasite transmission studies using membrane fed ticks. Voigt *et al* (1993) transmitted *T. mutans* and *C. ruminantium* to *Amblyomma variegatum* ticks and Inokuma and Kemp (1998) transmitted *Babesia*

bigemina to *Boophilus microplus* ticks. *T. parva* has been successfully transmitted by *R. appendiculatus* using a membrane feeding system (Waladde *et al* 1993, reviewed in Waladde *et al* 1996). The nymphal attachment levels were low at around 30% and the final engorgement weights were half those of cattle or rabbit fed ticks. The moulting success was lower with membrane fed ticks as were the adult infection levels compared with those that fed on equivalent parasitaemias in cattle. Because ticks take so long to engorge, erythrocyte sedimentation was found to be a problem if the blood was not replaced every 4 hours, making the technique very labour intensive (Waladde *et al* 1993).

Arthropod immunity

There are a number of factors within arthropods that can control microbial infection. These include the action of proteolytic enzymes, phagocytosis, encapsulation, lectins, lysozyme, antibacterial peptides, defensins and cecropins.

The extended period of time hard ticks take to feed in comparison with dipterans is one of the first major differences that would affect parasites within the bloodmeal. *Theileria* gametes fertilise in the gut lumen, after which the zygotes penetrate the digestive cells to continue their development. Gametes that remain in the lumen too long are likely to be agglutinated and then digested (Schein *et al* 1977). Blood digestion in ticks differs from insects in many ways. Insect digestion is a very rapid process that occurs in the gut lumen and involves many trypsin-like enzymes with a pH optimum above 7.0. Tick digestion on the other hand is very slow and occurs intracellularly in the gut digestive cells that contain aspartic proteases with a pH optimum of around 3.0 (Vundla *et al* 1992). The pH of the gut contents is around 6.5, a value at which the intracellular proteases referred to are inactive. Digestion starts in ticks at Stage 2 and 3 of feeding, but by the end of the expansion phase the proteolytic activity is only around 10% of its peak activity (Friedhoff 1990). Walker and Fletcher (1987) showed that specialised secretory cells in the gut of *R. appendiculatus* secreted acid phosphatase into the gut lumen during stage 2 of feeding, but not at the rapid engorgement phase when the majority of the bloodmeal

is imbibed. Schein et al (1977) reported that only gametes ingested at the final stage of engorgement went on to further development in the tick, as those ingested earlier were destroyed. The acid phosphatase secreted into the lumen could play a large part in preventing the survival of piroplasms and gametes ingested during the early stages of feeding.

Haemocytes are arthropod blood cells that circulate in the haemolymph. They constitute one part of the cellular defence capacity of ticks. They ingest, or form capsules, around foreign material and are involved in wound healing. It is thought that haemocytes are also involved in the production, storage and secretion of non-cellular mmune compounds which are associated with haemolymph coagulation, humoral encapsulation, melanisation, opsonization, self / non-self recognition and perhaps even cellular co-operation between different compartments within the tick (Lackie 1988). Three main types of haemocyte have been identified in studies involving female *Ixodes scapularis* ticks that simply classified their morphological characteristics (Zhioua *et al* 1996). Haemocytes in *I. ricinus* ticks were shown to have functional significance in clearing bacterial infections injected into the tick haemocoel (Kühn and Haug 1994) and in clearing naturally acquired *Borrelia burgdorferi* infection (Kühn *et al* 1994). Kühn (1996) showed an inducible accumulation of haemocytes in *I. ricinus* in response to wounding and during a bloodmeal, which has obvious implications for any parasites within the blood. An effective immune response has been demonstrated in the mosquito *Aedes aegypti* that showed a significant increase in the number of haemocytes produced in response to inoculated filarial nematodes (Christensen *et al* 1989). Eggenberger *et al* (1990) demonstrated that piercing the integument and implanting a small shard of epoxy plastic known by the trade name 'Araldyte' into the haemocoel of *D. variabilis* ticks resulted in haemocytic encapsulation. The encapsulation was biphasic, the first phase exhibited a recognition response and the second involved capsule formation.

Lectins are large proteins or glycoproteins that agglutinate cells and/or precipitate complex carbohydrates. Even though they have been isolated from a wide variety of

natural sources, including seeds, fungi, bacteria, body fluids of invertebrates and mammalian cell membranes, the precise physiological role of lectins in nature is unknown. However, it is clearly established that they are associated with arthropod immune systems (Renwrantz 1983) and bloodmeal digestion (Volf and Killick-Kendrick 1996). Grubhoffer *et al* (1991) showed that lectins in insects were important for binding sugars like sialic acid that enabled haemocytes to phagocytose bacteria. He also demonstrated their role in opsonisation and cytolysis. Welburn *et al* (1989) showed that lectins were responsible for killing trypanosomes in the guts of several *Glossina* sp. It was concluded that lectins were produced as a consequence of bloodmeal ingestion. Trypansosomes disappeared more quickly from the midguts of lies that had previously fed on a bloodmeal than from flies fed as tenerals. Lectins have been found in gut extracts from *I. ricinus* ticks and have been implicated in the spirochaete invasion process of the gut epithelial cells (Uhlír *et al* 1996). They have also been found in extracts of gut, haemolymph and salivary glands of *R. appendiculatus* (Kamwendo *et al* 1993). Kamwendo *et al* (1995) demonstrated that lectin inhibitory sugars infused into a nymphal feeding site on a *T. parva* infected calf had no effect on the resulting adult tick sporoblast level. Infected adult ticks feeding on a rabbit that had the feeding site infused with lectin inhibitory sugars developed significantly increased levels of sporoblast infection compared to controls, suggesting that lectins influence the development of *T. parva* in the tick salivary glands.

Melanins are an important group of pigments formed by the oxidation and polymerisation of phenols, such as tyrosine and dopa by enzymes collectively referred to as phenoloxidases. The importance of the phenoloxidase system and its role in encapsulation in vectors in relation to the parasites they transmit has been reported on few occasions. This is surprising considering Ratcliffe *et al* (1984) demonstrated the role of prophenoloxidase activation in non-self recognition and cell co-operation in insect immunity. Gregório and Ratcliffe (1991) showed that *Trypanosoma rangeli* was able to suppress the phenoloxidase encapsulating ability of its natural host *Rhodnius prolixus*, but was unable to suppress the same system in an

unnatural host, *Triatoma infestans*, in which the parasites were all readily killed by encapsulation. Zhioua *et al* (1997) were unable to detect phenoloxidase activity in unstimulated *A. americanum*, *D. variabilis* and *I. scapularis* unengorged female ticks. However, they did not check for the presence of prophenoloxidase inhibitors as have been found in the haemolymph of certain insect species (Tsukamato, 1992) or investigated whether the prophenoloxidase was present in an inactivated form as have been reported for other species (Smith and Söderhäll, 1991). The choice of unstimulated ticks was questionable for this investigation, as even physical damage to the integument may have induced a response as has been mentioned previously with haemocyte activation. Blood feeding may also have been an appropriate trigger as shown by the induction of lectin activity in *Glossina morsitans* (Abubaker *et al* 1995). Arthropods respond to bacterial challenge by the systemic production of a rapid and relatively short-lived profile of potent anti-bacterial peptides that have a broad spectrum of activity against prokaryote and even certain eukaryotic organisms. The cecropins are active against Gram-positive and Gram-negative bacteria and are thought to form channels in the bacterial membranes. Other small peptides have been found to be bacteriocidal or bacteriostatic against Gram-negative bacteria only, whereas the defensins, the most widespread group of peptides found in insects are active only against Gram-positive bacteria. These molecules are manufactured in the fat body and some blood cells. They accumulate in the haemolymph of the insect where they exert their effect against invading organisms. The production of the peptides can be induced not only by bacterial or parasitic entry, but by mechanical damage to the insect. In addition, blood coagulation and melanisation occur at the site of injury as a result of very rapid proteolytic cascade mechanisms. Arthropod proteolytic cascades show significant structural and functional similarities to mammalian acute-phase proteins which induce cytokine expression (Hoffmann *et al.* 1996) (Acute phase proteins appear in the blood in increased amounts shortly after the onset of infection or tissue damage, and serve to counteract some of the effects of tissue damage). Antibacterial peptides induced in *A. aegypti* resulted in significantly lower infections with *Brugia malayi* at low and medium microfilaraemias (Lowenberger *et al* 1996). The mosquito is susceptible to infection with *B. malayi*,

but refractory to *B. pahangi*, but neither of these infections on their own resulted in any antibacterial peptide RNA transcription. They concluded that the defensin production initiated by bacterial inoculation coincidentally affected the nematodes, and that the immunity responsible for refractoriness to *B. pahangi* must rely on factors other than antibacterial peptides. Johns *et al* (1998) investigated the antibacterial immunity of *Dermacentor variabilis* ticks. They showed a very rapid elimination of the bacteria, with complete clearance observed at 72hr post inoculation. During this period, the number of haemocytes increased as the number of bacteria decreased (sham injected controls did not show a significant increase, proving the rise in haemocyte numbers was due to bacteria and not a wounding response). A non-cellular response was enhanced in bacterially challenged ticks compared to controls. Initial investigations revealed that the protein they isolated, may have been a defensin.

Vernick *et al* (1995) investigated mosquito defence mechanisms against different *Plasmodium* species. They used different strains of *A. gambiae*, one of which was susceptible to infection and the other which was refractory. The refractory strain killed the migrating ookinete in the cytoplasm of the gut epithelial cells. Host cells containing dead or dying parasites showed pathogenic signs of vacuolation. The immune mechanism responsible for these effects was not known although the mechanism was linked to the mosquito esterase allele *Est A* (Vernick *et al* 1989).

Arthropod pathology

Even though parasite induced pathology in insects has been recognised for many years, literature has been relatively scarce on the subject until recently. Manson-Bahr (1912) (quoted by Kershaw *et al* 1953) reported *Wuchereria pacifica* induced mortality in *Aedes pseudoscutellaris* populations. He noted that levels of mortality increased markedly in warmer months of the year, which allowed parasite numbers to increase to unsustainable levels in the mosquitoes. Christensen (1978) had reported a similar effect of *D. immitis* on *Aedes trivittatus* in that mosquitoes could tolerate up to 15 microfilariae, but 16 or more caused rapid, high mortality. He noted it was rare to find mosquitoes that contained more than 16 microfilariae, even at high

microfilaraemias and suggested a mechanism which controlled parasite numbers within the vector to a mutually acceptable level for host and parasite. In a review article by Kershaw *et al* (1953) it was reported that *Dirofilaria immitis* infected *Aedes aegypti* also showed a considerably reduced survival compared to uninfected controls. They noted that two main peaks of mortality arose in relation to the parasite development. The first was when they were present in the gut, between days 0 to 5 post engorgement and the second was when the parasites were present in the haemolymph and salivary glands of the mosquitoes, up to day 14. In between these two periods, development in the Malphighian tubules cells seemed to cause the mosquitoes no detrimental effects.

Schaub and co-workers have produced a large amount of literature on the pathological effect of the homoxenous flagellate *Blastocrithidia triatomae* on its reduviid bug vector *Triatoma infestans*. The bug is responsible for transmitting *Trypanosoma cruzi*, the causative agent of Chagas disease in man and the German workers' interest in the *B. triatomae*/*T. infestans* relationship was of biological control of the vector.

Schaub and Meiser (1990) showed the intestine of the reduviid bug was damaged by infection with *B. triatomae*. Dispelled rectal fluid was red, which is normal in some haematophagous insects like *Anopheles stephensi* (Briegel and Rezzonico 1985) but is only seen in reduviid bugs after coprophagic infection with *B. triatomae* (Schaub 1988, Schaub and Breger 1988). Red coloured intestines were seen which indicates abnormal haemoglobin digestion, as the control intestines were dark brown and finally the haemolymph in a small number of bugs had a reddish tint because it contained haemoglobin. This shows the intestine must have been perforated and allowed leakage of digestive contents. Schaub (1990) also showed that membrane feeding the bugs with *B. triatomae* cysts in blood induced digestive disturbances, retarded larval development and greatly increased mortality in later instars compared to controls. The physical nature of the pathology was elaborated in electron

micrographs (Jensen *et al* 1989, Schaub and Neukirchen 1992). Several examples of pathology were seen, mainly to the gut epithelial cells including: reduced microvillar height and numbers on the inner membrane of the cells, reduction in mitochondria, widespread vacuolation and occasional host cell lysis. Very dense accumulations of flagellates were shown over the microvillar border that displayed three modes of attachment. In areas where microvilli were still present, the expanded flagella of the parasites interdigitated between them and in some cases actually penetrated the microvillar membrane. In areas where microvilli were absent (as a consequence of infection) enlarged flagella attached directly to the epithelial membrane. After parasite detachment, the cells of the epithelia were often seen to have lysed. Retarded larval development and later instar mortality was shown to be directly related to parasite density in the bloodmeal (Schaub *et al* 1992).

Unlike *Theileria*, the *Babesia* lifecycle involves schizogonic multiplication in the tick as opposed to the mammalian host. Transovarial transmission also occurs in the tick and enormous numbers of vermicules can infect the developing eggs at this stage. Because the parasites increase in number throughout the tick moult, there are usually many more parasites present than at the equivalent stage of moult in a *T. parva* infected *R. appendiculatus* tick. However, it is clearly detrimental for the continuance of the *Babesia* lifecycle to overwhelm a tick host so that it suffers excessive morbidity or mortality. *Babesia bigemina* piroplasm levels in excess of 20% infected erythrocytes were linked to pathological effects on the *Boophilus microplus* ticks studied by Riek (1964). Around 90% of the females suffered mortality soon after engorgement and this was preceded by the haemolymph turning red. The gut epithelial cells had been damaged by the parasites causing undigested blood to escape. The surviving ticks laid small egg batches, most of which were infected. The pathology was only seen when ticks fed on an animal showing clinical signs of babesiosis with high parasitaemias. Ticks engorging on animals with asymptomatic infections and very low piroplasm parasitaemias produced no adverse effects and resultant tick infections were low. Agbede *et al* (1984) studied the development of *Babesia bovis* in the gut cells of *B. microplus*. They noticed a

parasite-induced change in host cell morphology as early as day 4 post detachment of the tick. Infected tick cells had hypertrophied nuclei during zygote maturation within the cells, and parasitised cells were less basophilic compared with uninfected cells, and showed clear signs of degeneration. Kinete formation from day 4 onwards resulted in host cell destruction with complete degeneration of organelles and greatly reduced endoplasmic reticulum.

Very heavily *B. bigemina*-infected larval, nymphal and adult *Boophilus decoloratus* were studied by Potgieter and Els (1977). They reported extensive host cell damage which was unsurprising because the parasite densities in their sample ticks were such that kinetes were so packed into some gut epithelial cells that host cell cytoplasm was barely recognisable. Examples of curled up parasites at the zygote to kinete developmental stage were seen free in the haemolymph. They had presumably burst out of gut epithelial cells and were continuing their development in an extracellular environment. The authors did not report levels of tick mortality or resulting salivary gland infection quanta, but it is likely that the level of pathology reported, combined with the extent of the parasite burden would have resulted in extensive losses.

Reduction in reproductive capacity is one of the most common ways in which arthropods are affected by parasites. In the case of insects, the fecundity is defined and assessed in terms of the number of gametes produced, whereas fertility refers to the number of viable offspring produced (Clements 1992). The term parasitic castration was introduced by Noble and Noble (1971) who described it as ''destruction or alteration of gonad tissue by the parasite'' and by Malek and Cheng (1974) as a total or partial inhibition of gamete formation in hosts by parasites.

In contrast to the results by Riek (1964), Gray (1982) demonstrated in *B. microplus* ticks, reduction of survival and reproductive ability induced by *B. bigemina* when the ticks engorged on blood with piroplasm parasitaemias less than 1.5%. Reduced egg batch weights and percentage hatching as well as mortality levels as high as 93 to 100% were seen in tick batches which detached from calves showing piroplasm

parasitaemias of 9 and 10%. Riek (1964) reported that the vector/parasite interaction was quite specific and certain strains of *B. bigemina* would only develop in particular strains of *Boophilus*. The differences noted between the results of the different investigations are very likely a consequence of strain differences. It is important to be very aware that laboratory strains of tick and parasites may have adapted to each other through continual passage and may gradually become less representative of those in the field and in other laboratories.

Ham and Gale (1984) found that filarial nematode infections of British and African blackflies (*Simulium* sp.) resulted in smaller egg batches. Further research showed that ovarian uptake of vitellogenin was affected as early as 24hr post-infection with microfilariae (Renshaw and Hurd 1984a and 1984b). A significant dose effect was not observed in this study as a single microfilaria was sufficient to produce a substantial effect. It is difficult to see an advantage for parasites of this effect on their hosts, as it will reduce potential future host populations. Using a model system with the tapeworm *Hymenolepis diminuta* in its beetle, intermediate host *Tenebrio molitor*, Hurd and Arme (1986) reported reduced fertility as a result of infection and addressed this question. They suggested that at the time of rapid parasite growth and development, a reduction in the energy and nutrient commitment to egg production in female hosts may reduce the adverse effects of the parasites. This idea is further explored by Hurd (1998) in the light of recent findings and related to the evolutionary implications for a range of arthropod host and parasite species.

Transgenic arthropod vectors

The New World screwworm was successfully eradicated from Mexico (Krafsur *et al* 1987) and Libya (Lindquist *et al* 1992) by the release of sterile males. Five day old larvae were sterilised with radiation and after adult emergence, huge numbers of both sexes were released into the environment. The success of this strategy encouraged interest in the concept of controlling insect pests or disease vectors by genetic manipulation. Sterile male release is unlikely to be appropriate for eradicating all insect vectors. In an article discussing the merits of producing transgenic mosquitoes

as a means of malaria control, Curtis (1994) commented that mosquito control by the release of sterile males was unlikely to be sustainable. An influx of wild-type mosquitoes from surrounding areas would be anticipated. A more sustainable strategy would be to introduce a transgenic, *Plasmodium*-refractory strain of mosquitoes that would outcompete the wild-type susceptible mosquitoes through natural selection and eventually dominate the population. Genes can be inserted into organisms in a number of ways. Transposable elements, or transposons, are DNA sequences that can be inserted into many different sites in the host's chromosomes. They have been successfully used to insert genes into *Drosophila melanogaster* (Bellen *et al* 1989) and are being developed to allow genetic insertion into other insect species (O'Brochta and Handler 1988). Viruses are a major source of material from which gene vectors are developed. Retroviral vectors are naturally infective and do not need to be delivered to the target cells, which is a technical difficulty associated with transposon vectors (O'Brochta and Atkinson 1997). *Wohlbachia* can be transmitted through the egg cytoplasm in *Culex* mosquitoes, *D. melanogaster* and other insects (Stevens and Wade 1990). They are also known to be transmitted transovarially in ticks (Diehl and Aeschlimann 1982). Collins (1994) (quoting O'Neill - personal communication) speculated that arthropod vectors could be infected with a genetically manipulated virus and a *Wohlbachia* strain in the same cytoplasm so that the virus could be maternally transmitted and fixed in wild populations. The genetic manipulation of vectors does not however, receive universal support as a realistic means by which vector-borne diseases will be controlled. Spielman (1994) put forward several reasons why antimalarial research should not focus on transgenic mosquitoes. The vector competence of mosquitoes contributes relatively little to the force of malaria transmission - the longevity of the mosquitoes is considered much more important. An abundant long-lived insect vector that focuses its feeding on a particular host species may produce numerous secondary infections, even if its competence were very small. He disputed that transgenic mosquitoes would replace a wild-type population because anopheline mosquito populations are not density dependent (apart from the larval breeding sites). He stated that release of large numbers of transgenic, malaria refractory mosquitoes would simply result in additional mosquitoes that were not vectors for the disease.

Another, more practical objection raised, regarded the ethics of releasing large numbers of haematophagous insects. Although a species may have been made refractory to one infectious agent, it is possible they would be able to transmit other organisms. It may also be very difficult to convince local people of the theoretical benefits of mass-release of arthropod pests.

The main aim of the experiments carried out for this thesis was to try to better understand the nature of the *T. parva*/*R. appendiculatus* association. One of the main problems with obtaining epidemiological data on *T. parva* infection in ticks is that the majority of ticks die before they are returned to the laboratory for processing, making them unsuitable for dissection. A PCR technique to detect *T. parva* in alcohol preserved ticks could allow a great deal more epidemiological information on ECf incidence to be extracted from field collected ticks. Evaluating the PCR in the field, in conjunction with conventional techniques would then be an appropriate test. Only a small proportion of *R. appendiculatus* adults assessed from most field collections are infected with *T. parva*. Very little is known about the dynamics of *T. parva* in *R. appendiculatus* during the tick moult. In particular, establishing which parasite form suffers the greatest mortality in the tick might be of great use in developing a transmission-blocking vaccine. There is an equal dearth of information on the pathogenic effects of *T. parva* on ticks. Addressing this issue would be very important in relation to epidemiological studies in endemic areas as tick population dynamics could be greatly affected by small, parasite induced changes in fecundity and mortality. Because of the huge variability in the quanta of *T. parva* forms in infected ticks, a large numbers of ticks would have to be examined microscopically for accurate data to be generated. Examining sectioned ticks, stained with conventional histological stains is very time consuming and can be subjective. A DNA-based *in situ* hybridisation technique to detect *T. parva* forms within sections if infected ticks could improve the accuracy of, and potentially greatly increase the processing time of this approach. This thesis sought to address these issues.

CHAPTER 2

General materials and methods section

Licences

All animal experimentation was conducted under the sanctions of the Animal (Scientific Procedures) Act 1987 of the U.K.

Tick rearing procedures

Procedures regarding the rearing of ticks were described by Bailey (1960). Female, New Zealand White rabbits weighing 2.5 to 3.5 kg were used for tick feeding purposes. The ears were clipped of hair from just above the base and a cloth bag was attached with zinc oxide tape. A large area in the middle of the back was shaved and a cotton sleeve was attached around the torso with internal hook fastener strips ('Velcro'') and elastic cord drawstrings. A plastic Elizabethan Collar was attached around the rabbit neck to prevent removal of the bag by the rabbit. Larvae were fed exclusively in ear bags as they tended to escape from the cotton sleeves tied around the body. Nymphs and adults were fed from either position depending on circumstance. To remove the engorged, detached ticks from the ear bags, the rabbits were tightly wrapped in a cloth and inverted over a collecting container. Gently shaking the bags resulted in the ticks falling out. The cotton sleeves around the body were removed and the detached ticks, which were invariably under the belly, were brushed out into a deep tray. The cotton sleeves were used for nymph and adult feeds as routine colony maintenance as larger numbers of ticks could be placed in them and they tended to be less stressful for the rabbits than ear bags. Ear bag feeds were employed if it was important to have two separate batches feeding on the same host. Adult ticks could be placed on the same feeding site as larvae or nymphs, but larvae and nymphs were never mixed together as they were difficult to separate after they had engorged and detached. If adults were being applied, the males were usually placed in the bags a day before the females as this tended to improve the attachment and feeding performance of the females.

Engorged ticks were cleaned with running, tepid water (to remove tick faeces etc.) through a fine mesh sieve and dried with blotting paper. The batches were weighed, recorded and divided into appropriately sized amounts in universal tubes closed with cotton wool bungs. Around 100 adults, 500 nymphs and 1000 larvae were stored in each tube. If too many ticks were stored, the tubes became contaminated with a profuse growth of mould and algae.

Humidity levels were maintained by saturated salt solutions (Buxton and Mellanby 1934) of sodium tartrate (85 - 92%) or potassium chloride (85%). For moulting, the ticks were placed in the incubators under the following conditions:

Table 2.1. Storage conditions for *R. appendiculatus* instars.

Tick Stage	Temp. (^{0}C)	hr. light/dark	r.h (%)	No. of days
egg laying females	28	18 / 6	85 - 92	60
engorged larvae	28	18 / 6	85 - 92	15
engorged nymphs	28	18 / 6	85	30

Calf infection and monitoring

Ground up tick supernatant (GUTS) stabilate

The methods described by Cunningham *et al* (1973) were followed during these procedures. Infected adult *R. appendiculatus* were fed on a rabbit for 4 days to allow sporozoite development to occur and then removed. They were surface sterilised by being washed with 1% benzalkonium chloride (Roccal, Winthrop Laboratories, England) and then washed three times in 70% alcohol. They were transferred to a sterile vial during the second wash in alcohol. The ticks were then washed in Eagles Minimum Essential Medium (MEM) with Hanks salts, penicillin - 200 iu/ml, streptomycin - 200 μg/ml and nystatin - 100 μg/ml. They were left in a fourth wash of medium for 10 min and then transferred to a sterile mortar with MEM/bovine

plasma albumin (BPA) at 3.5% (Armour Fraction V). The contents of the mortar were ground gently with a sterile pestle and the supernatant was constantly removed. Medium was added to the mortar until the supernatant appeared to be less cloudy with tick debris. It was important that the ticks were ground only enough to crush them to release the sporozoites from the salivary glands. Excessive grinding was avoided as it contaminated the sporozoite suspension with tick debris. The supernatant was centrifuged at 100 **x g** for 5 min and then aseptically removed (it contained a sporozoite suspension at this stage). The supernatant was then filtered through a sterile 8μm high-porosity cellulose acetate filter (type MF, Millipore Corporation). A fifty to one hundred microlitre volume of each batch was placed in a cytocentrifuge funnel and centrifuged at 100 **x g** for 10 min onto microscope slides. The slides were then stained in 5% Giemsa's stain for 40 min and examined by light microscopy with the x 100 objective for the presence of sporozoites.

Cryopreservation

Sterile glycerol (analar grade), prewarmed to 37^{0}C, was added to 3.5% MEM/BPA to make a solution of 15% glycerinated cryoprotectant medium. An equal volume of this solution was added slowly to the GUTS stabilate and was gently pipetted. The sporozoite suspension was left to equilibrate at room temperature for 30min after which it was aliquoted into 1ml vials. The vials were stored at -70^{0}C overnight and then transferred to liquid nitrogen the following day.

Resuscitation

After removal from liquid nitrogen, the vials were thawed rapidly in a water bath at 37^{0}C and left to equilibrate at room temperature for 30min.

Sporoblast abundance was calculated by examining stained preparations of fifty randomly sampled ticks from the batch used to make the GUTS stabilate. The sporozoite suspension was diluted in medium and cryoprotectant until a specific dilution was reached based on the results of previous cattle infections i.e. if the calf died very rapidly the stabilate was further diluted until a more moderate clinical

reaction was seen. The infection data for the ticks used to make *T. parva* Muguga Stabilate 71 were 95% infected with an average of 121 sporoblasts per tick, whereas the ticks used to prepare *T. parva* Marikebuni Stabilate 72 were 95% infected with an average of 78 sporoblasts per tick. Tick equivalent (t.e) was used as a quantitative measure of sporozoite content in a stabilate inoculation in this laboratory. *T. p* Muguga Stabilate 71 was inoculated at 1.25t.e which was equivalent to 150 sporoblasts and *T. p* Marikebuni was inoculated at 2t.e which was equivalent to 156 sporoblasts.

Calves used were generally 3 to 9 months of age and between 75 and 120 kg. They were either Friesian or Holstein-Friesian breeds. A generous area was clipped of hair over the left and right prescapular lymph nodes (RPG) and the jugular vein on both sides of the neck as well as both ears and small areas on the neck (for tick feeding purposes). After the stabilate vials were removed from liquid nitrogen and resuscitated (see above), the vial contents were thoroughly mixed through a syringe and inoculated by subcutaneous injection above the RPG. Rectal temperatures were recorded daily from day 0 and needle biopsies of the RPG were taken daily from day 5 onwards and the blood daily from day 7.

Antibiotic Administration

Tetracycline (Engemycin 10% DD, Intervet UK Ltd) was usually administered to the calf in an attempt to obtain high piroplasm parasitaemias. When the calves' temperature exceeded 40^0C, the oxytetracycline was administered at a dose of 10mg/kg body weight. The drug was given to reduce pathogenesis, thereby preventing early calf death, and allowing the piroplasm parasitaemia to increase over the period in which ticks were likely to be feeding.

Other methods to increase the probability of high *T. parva* piroplasm parasitaemias have previously included: infecting splenectomised calves (Voigt and Büscher, ILRI, Kenya, unpublished communication) and administering classical corticosteroids (to suppress the immune system) such as Dexamethosone (R. Payne; NVRC, Kenya,

personal communication), although neither of these methods were used in the experiments described here.

From day 5 post-infection onwards, lymph nodes were gently palpated to check their overall size, which was recorded. The size grading is on an arbitrary 4 point scale of 0 to 3, with 0 represented a small, uninfected node whereas 3 represented a large, immunologically active node (≤ 15cm diameter x 8cm deep - approx.). To sample the lymph node, the overlying skin was then wiped with 70% alcohol and with the node being held firmly but gently in one hand, a one inch long, 19G needle was inserted. The needle was either slowly rotated or squeezed with a finger over the end to increase and release the pressure until fluid was seen in the reservoir of the needle. The needle was removed and the lymph node was massaged and wiped over with alcohol. The fluid in the needle was applied to a slide, smeared with a spreader and allowed to dry. The slides were fixed in methanol for 3 min. before being placed in 5% Giemsa's stain, diluted in Giemsa buffer, pH 7.4, for 40 min. The white blood cells tend to be pushed to the top of the smear so that examination of the 'tail' was carried out with a x50 and x100 objective. The level of macroshizont infection was ranked on a 4 point scale; 0 (uninfected), + (<1% infected cells), ++ (1 - 5% infected cells), +++ (>5% infected cells).

Blood samples were taken daily from day 7 onwards. Around 10mls were collected from the jugular vein in 4.5ml K3 EDTA coated vacutainer tubes (Becton Dickinson, England) or plain 10ml vacutainer tubes (Becton Dickinson, England) that had 1ml of sterile acid citrate dextrose (ACD) anticoagulent added. A small volume of blood was spotted onto a slide and smeared. The fixing and staining protocol was the same as described above. Examination was carried out with a x100 objective on areas with evenly distributed erythrocytes. The *T. parva* parasitaemia values were established after counting 1000 cells in each of five separate areas of the slide with an average value being established to one decimal place.

Tick infections

Cloth bags were stuck onto the clean and intact hair around a small, clipped area of skin using a water-based latex glue (Copydex, Pritt). Universal tubes containing ticks were placed in the bags from day 7 to 9 (exact day depended on interpretation of clinical signs) and then again two days later to step the application. The empty vials were removed the next day and the bags were checked daily to monitor the attachment levels and feeding performance.

Assessing tick salivary gland infections

The procedures described by Blewett and Branagan (1973) were followed. The salivary glands of adult ticks were most commonly assessed for infection, although nymphal glands were also examined. The ticks were lightly embedded in wax on the bottom of a Petri-dish which was then overlaid with 1% saline (sodium chloride) solution. The scutum was removed using No. 11 scalpel blades (Swann-Norton ®, England) and the Malphighian tubules and tracheae removed. The gut was gently pulled away at the anterior end revealing the salivary glands. The glands were removed by their anterior end and placed on a slide in a small drop of a 1% bovine plasma albumin (BPA) (Sigma) solution to aid gland adherence. They were teased out with dissecting forceps and dried on a plate heated to 40^0C.

Preparation of methyl green and pyronin stain

A 2% (w/v) methyl green stain (Sigma, England) solution was prepared in water. An equal volume of chloroform was added and vigorously shaken in a separating funnel. The methyl violet contaminated chloroform was discarded and the process repeated until the chloroform was clear. This represented a purifying step for the methyl green. Pyronin Y (Sigma) was diluted in water to make 100ml of a 5% (w/v) stain solution. Eight millilitres of pyronin solution was added to forty millilitres of methyl green solution, and made up to 500ml with distilled water. The stock stain solution was kept at 4^0C in the dark and was stable for six months. Acetic buffer solution was prepared by adding distilled water to glacial acetic acid to give 200ml of a 0.6%, pH 4.8 solution. 300ml of a 9.5 x 10^{-2}M anhydrous sodium acetate solution was prepared

and added to the first solution, giving 500ml of acidic buffer which was stored at 4^0C. An equal volume of acidic buffer was added to stock stain solution to give the working MGP stain.

Staining procedure

The slides with the dried salivary glands were fixed in 90% ethanol for 1 min and placed in MGP stain solution for 10 min. Slides were then dehydrated in 90% ethanol for 1 min, absolute ethanol for 2 min and then cleared in m-xylene for 2 min. After the slides were overlaid with plastic mountant (DePeX, Gurr, BDH) and a coverslip, they were dried on a heated plate. Examination was carried out using a x10 and x25 objective.

Tick preparation

Unfed, unstimulated ticks were examined for infection in one experiment. The sporonts are very small at this stage and extremely difficult to distinguish with MGP. The technique was only used in one experiment where it was important to compare the infection assessment by PCR and microscopy on unstimulated ticks, as they were most representative of field collected ticks removed from vegetation.

The ticks could be partially fed to activate their salivary glands and the sporoblast infections within them (Purnell and Joyner 1968), that made the parasite masses considerably easier to see when stained. Adult ticks were fed for 3 to 4 days on rabbits before being forcibly removed and nymphs were fed for 2 days. Feeding them for longer periods results in some or all of the sporoblasts developing into sporozoites and being passed out of the tick in the saliva.

Incubation of adult ticks in a humid atmosphere at 37^0C for 5 days encouraged sporoblast maturation. This technique was slightly less effective than partial feeding, but was more consistent and did not require the use of animals. Because of the large numbers of tick batches used over the course of the study and for technical reasons, this approach was adopted for the majority of adult salivary gland assessments.

CHAPTER 3

Theileria parva detection in *Rhipicephalus appendiculatus* using the Polymerase Chain Reaction

The aim of this chapter was to develop a PCR capable of detecting *T. parva* in alcohol preserved *R. appendiculatus*, representative of those collected and stored from field studies.

Introduction

Randolph and Craine (1995) showed that even in a simple mathematical model to evaluate the risk of tick-borne disease transmission, there are three fundamental parameters that need to be determined; namely, the infestation level of ticks on the host population, the prevalence (and to a slightly lesser extent, the abundance) of infection in the ticks, and the susceptibility of the host population to the disease.

There are a number of different techniques which can be used to confirm the identity and quantify a pathogen in a vector. Purnell *et al* (1973) cut and stained sections of adult salivary glands and whole nymphal *R. appendiculatus* infected with *T. parva* to compare parasite development during tick feeding. In a study to assess the prevalence of *Cowdria ruminantium* infection in field collected *Amblyomma hebraeum*, Norval *et al* (1990) fed small groups of 2 to 10 ticks on susceptible sheep which were monitored for infection. Unfed, field collected ticks can be partially fed in the laboratory, processed for GUTS stabilate which can be inoculated into *in vitro* cultures of susceptible host cells using the method developed by Brown *et al* (1979). Antibody detection techniques such as those developed by Kimber *et al* (1973) have been used to detect *T. parva* in whole salivary glands. More recent techniques have involved the use of radiolabelled DNA probes to detect *T. parva* in *R. appendiculatus* salivary glands that were lysed onto nylon filters (Chen *et al* 1991).

Because of the low prevalence and abundance of *T. parva* in field ticks (Walker *et al* 1981) a large number of ticks have to be examined before accurate data can be

obtained. Assessing tick infections by microscopy necessitates the dissection of live ticks. Field collected ticks are often kept in containers for a number of days before it is possible to process them in a laboratory. As a result there is usually high mortality, particularly of ticks removed from animals as opposed to those collected from vegetation, as these are even more difficult to keep alive. In many cases, the ticks are placed directly in alcohol for later identification and counting. Alcohol fixation makes the ticks unsuitable for dissection, thereby removing the option of whole gland examination. A technique which could detect infection in these samples and all the archived, alcohol preserved material available would be very valuable to improve the accuracy of epidemiological models.

R. appendiculatus is a vector for *T. taurotragi* as well as *T. parva*, and field ticks are often infected with both species (Morzaria 1989). Morphological discrimination between sporoblasts and sporozoites of two species in tick salivary glands is very difficult (Bishop *et al* 1994) making alternative techniques essential if accurate data for quantitative epidemiological studies and mathematical models is to be obtained. Detailed images and descriptions of *T. taurotragi* (Young *et al* 1980) and *T. parva* (Mehlhorn *et al* 1978) in *R. appendiculatus* are available and could be used as a guide if salivary glands or whole ticks were sectioned and stained (Purnell *et al* 1971) as the sporont stages of the two species are quite different (Young *et al* 1980). A more recent approach to discriminate the two species in the salivary glands used ribosomal RNA probes after the dissected glands had been squashed onto nitrocellulose membranes (Bishop *et al* 1994). However, neither of these approaches would be appropriate for dealing with the large number of field ticks that would have to be processed. A polymerase chain reaction (PCR) technique which could differentiate between the two species in dissected salivary glands, or more conveniently, whole ticks would be extremely valuable.

PCR is an *in vitro* technique that allows the amplification of a specific deoxyribonucleic acid region that lies between two regions on a known DNA sequence. The amplification is achieved by using oligonucleotide primers which are

complementary to the ends of a defined sequence of DNA template. The primers are extended on single stranded, denatured DNA by a DNA polymerase in the presence of deoxynucleotide triphosphates under suitable reaction conditions. This results in the synthesis of new DNA strands complementary to the template strands. Strand synthesis is continued by repeatedly denaturing the double stranded template, cooling the reaction to allow primer annealing and primer extension at an appropriate temperature for the polymerase enzyme. Each new DNA strand synthesised becomes a template for further amplification cycles which leads to an exponential increase in the amount of specific template sequence. A PCR which amplified DNA from the TPR1 region of the *T. parva* genome has been previously described and was shown to be very sensitive and species specific (Bishop *et al* 1992). Because the TPR1 region is multicopy, the chance of primers annealing is greatly increased over sequences of lower copy number making it a particularly suitable target area for sensitive detection.

The first experiment used *T. taurotragi* and *T. parva* DNA in a PCR to establish whether a difference in the PCR products between the two species could be established. *T. taurotragi* DNA was not used in later experiments, but it was important to establish the specificity of the reaction.

The aim of the second experiment was to compare the *T. parva* prevalences obtained from the PCR and MGP stained salivary glands from a batch of experimentally infected ticks. Glands were dissected from ticks over a range of rising and falling piroplasm parasitaemias to establish a comparison over as wide a range as possible. It was necessary to establish the reliability and accuracy of the PCR by comparing it with the standard MGP method before it could be used on whole ticks in which there was no alternative technique for verifying the infection status.

The third experiment compared PCR and microscopy data for prevalence and abundance of infection in paired salivary glands from individual ticks. Batches were chosen over a well graded range of infectivity based on data obtained from the first

experiment. PCR amplicon intensity from one gland was correlated with the sporoblast count from the other. The amplicon intensity in this case represents a measure of prevalence and abundance.

The terms prevalence and abundance have been defined by the American Society of Parasitologists as follows (Margolis *et al* 1982): Prevalence (usually expressed as a percentage) is the number of individuals of a host species infected with a parasite species divided by the total number of hosts examined. Abundance is the total number of individuals of a parasite species in a sample of hosts divided by the total number of infected and uninfected hosts in the sample. As an example, a *R. appendiculatus* population harbouring *T. parva* infection with a 50% prevalence and an abundance of 25 means that 50% of the ticks are infected and each tick has an average of 25 sporoblasts.

The final experiment compared the effectiveness of the PCR when applied to unfed and partially fed ticks which represent those likely to be collected under field conditions. It was necessary to determine whether the presence of host blood within partially fed ticks was inhibitive to the PCR. Randomly selected ticks were picked from batches with low and high infections and split into two groups. One group was partially fed on rabbits to allow their sporoblast infections to mature while the other group were processed unfed to represent questing ticks.

Materials and methods

3.1. *Preparation of positive control material*

Positive control material can be generated from ticks, cell culture or blood samples infected with *T. parva*. To provide a very pure sample of *T. parva* DNA, piroplasms were purified from infected bovine blood.

A calf with a *T. parva* Muguga piroplasm parasitaemia in excess of 10% was bled to death under anaesthesia. Sterile one-litre bottles were prepared containing 100 ml of ACD. The blood was collected by anaesthetising the calf with pentabarbitone sodium

BP (Euthatal, Rhône Mérieux) at 0.2ml/kg body weight. The jugular vein was surgically exposed and swabbed with ethanol, clamped and then cut with a sterile scalpel. The clamp was released and the blood funnelled into the bottles that were continually agitated to ensure the ACD mixed thoroughly.

The piroplasm purification technique was adapted from a method by Sugimoto *et al* (1990).

The blood was centrifuged at 1000 **x g** for 10 min at 4^0C. The plasma and buffy coat were removed and the erythrocytes mixed with PBS at 4^0C. After washing the red cells twice in PBS, they were resuspended in three volumes of PBS and applied to a glass column filled with cellulose powder (Whatman Fibrous cellulose powder CF11, Mean particle size 50-350μm). A small amount of glass wool was lightly pressed into the bottom of the column before the cellulose powder was added. 40g of cellulose were used to filter 500ml of erythrocyte suspension. The cell suspension was filtered to remove contaminating white cells that were still present after buffy coat removal, as the white cells stuck to the cellulose matrix while erythrocytes passed through. The cell solution was slowly applied to avoid disrupting the powder and allowed to flow through the column into a beaker placed in an ice bath. The beaker was regularly agitated to avoid cell clotting. The cells were washed once in PBS and then suspended at a concentration of 50% in PBS. Three volumes of 0.083% ammonium chloride (NH_4Cl) at 37^0C were added to the cell solution for 5 min to lyse the erythrocytes. The solution was then cooled on ice and EDTA added to a final concentration of 5mM. The lysate was centrifuged at 2000 **x g** for 10 min at 4^0C and the supernatant carefully poured off. The pellet was washed 2 to 3 times in PBS until the supernatant was clear. Before and after every wash step, a 50μl volume of cell solution was removed, diluted with PBS, prepared in a cytological centrifuge (Cytospin, Shandon Ltd) and Giemsa stained to repeatedly check that no white blood cell contamination was present (as white cells do not lyse in ammonium chloride solution).

Eagles Minimum Essential Medium (MEM) (Life Technologies) was prepared under sterile conditions containing 3.5% bovine plasma albumin (MEM/BPA). Prewarmed glycerol was added to a final concentration of 15% (v/v), and mixed by very thorough pipetting. A volume of this solution, equal to that of the piroplasm pellet was added dropwise to the piroplasm solution and mixed by gently inverting the tube. The solution was left at room temperature for 30 mins to equilibrate. After aliquoting into 1ml vials, the tubes were stored at -70^{0}C overnight and the next day placed in liquid nitrogen.

When positive control material was required, a vial was removed from the liquid nitrogen and DNA extracted. It was found that this method provided a much longer lasting stock of material for control DNA than extracting a large amount at one time and storing it in H_2O or DNA elution buffer at -20^{0}C. This method was found to result in DNA degradation and the consequent loss of positive control amplicon strength within 2 to 3 months, presumably because of the presence of DNAses.

3.2. *DNA extraction*

DNA was extracted from the samples using the QIAgen Tissue Kit (QIAGEN Ltd) as per the manufacturer's instructions. Sample material was initially placed in 180µl of lysis buffer (Buffer ATL) in a 1.5ml microfuge tube. 200µl of blood and cultured cells were chemically lysed, but tick material required homogenisation with plastic micropestles (Eppendorf). QIAgen Proteinase K (PK) was added to a final concentration of 2mg/ml (60ma.u) and the samples incubated in a waterbath at 55^{0}C, with regular vortexing, for 1hr to overnight depending on the sample. Blood and cultured cells required the minimum period, whereas tick material, particularly whole ticks required a longer period for complete digestion. 200µl of buffer (Buffer AL) were added and after thorough mixing, the tubes were incubated at 70^{0}C for 10 mins. 210µl of ethanol were added and mixed thoroughly by vortexing to precipitate the DNA. The digested material was then removed from the tube and placed in the QIAgen column filter. To prevent blockage of the filter with large particulate material, the tick material in particular was briefly centrifuged beforehand and the

supernatant withdrawn. The chitinous exoskeletons were unaffected by the PK and were removed before further processing. The filter was placed in a 2ml collection tube provided with the kit and centrifuged for 1 min at 6000 **x g**. The collection tube and eluate were discarded. The filter was then placed in another collection tube and 500µl of washing buffer added before centrifugation. The procedure was then repeated, to further remove any protein which may have been adhering to the filter and to wash out salts present in the reaction buffers. After the washing steps, the filter was placed in a 1.5ml microfuge tube and 100µl of dH_2O heated to 70°C was added. The tube was incubated at 70°C for 5 mins and then centrifuged at 6000 **x g**. The eluate containing the DNA was then aliquoted into 20µl volumes and stored at -20°C.

3.3. *PCR method*

The PCR was adapted from a method by Bishop *et al* (1992).

100µl PCR reactions were carried out in 0.5ml microfuge tubes (Eppendorf). The reaction mixes contained 2mM Tris-HCl, pH 8.55, 5mM $(NH_4)_2$ SO_4, 100µM EDTA, 1mM 2-mercaptoethanol, 0.05% Thesit™, 5% glycerol (contained within Thermometic Reaction Buffer), 200µM each deoxyribonucleotide, 1µM each primer, 2.5mM $MgCl_2$, *Taq* DNA polymerase (1.25u/µl). 2.5µl of DNA solution was added per reaction. Reactions were overlaid with 50µl of fine mineral oil (Sigma) to prevent evaporation and denatured at 95°C for 2 min. The mixture was subjected to 30 cycles of denaturation (95°C - 1 min), annealing (60°C - 1 min) and extension (72°C - 1 min).

Examining PCR products

1.6% gels were made with molecular biology grade agarose (Sigma), 0.5 x Tris borate EDTA (TBE) (diluted from a 5 x stock - see Appendix 6) and ethidium bromide (final conc. 3.2µg/ml). 13µl of amplified products were mixed with 3µl of 6 x loading dye (Sigma) and loaded into the gel slots. The gel was run in 0.5 x TBE at around 80mV. Examination was carried out using a U.V transilluminator.

3.4. *PCR using* T. parva *and* T. taurotragi *DNA*

DNA was extracted from sporozoite stabilates. *Theileria parva* Muguga stock Stabilate 71 and a *Theileria taurotragi* stabilate (prepared by the National Veterinary Research Centre (NVRC), Muguga, Kenya) were used. DNA was extracted from the material using the QIAgen DNA extraction columns as previously described. A graded range of *T. parva* and *T. taurotragi* DNA concentrations was added to the PCR. The nucleic acid concentration was not determined spectrophotometrically because GUTS stabilates do not contain highly purified sporozoites. The extracted DNA would have contained a mix of *Theileria* and *R. appendiculatus* DNA. Stating the concentration of extracted DNA as if it was purely from *Theileria* could have been misleading.

3.5. *Calf and tick infection*

Calf 552 was infected above the RPG with 0.25t.e (30 sporoblasts) *T. parva* Muguga Stabilate 71. *Rhipicephalus appendiculatus* nymphs were applied (x 1000) in each of two earbags and a neck patch and they detached during a piroplasm parasitaemia which ranged from <0.1 to 2.4%. Detached, engorged nymphs were stored at 28^0C for 28 days until they moulted to adults and at 18^0C thereafter. The infection profile from calf 552 is shown in Appendix 1.

3.6. *Assessing tick infections with PCR analysis and microscopy*

Seven groups of ticks were selected from batches that detached from rising and falling parasitaemias. Ten ticks (5 males, 5 females) were included in two groups, giving a total of 140 ticks. Salivary glands from 70 ticks were assessed for *T. parva* infection by PCR and 70 by microscopy. The range of piroplasm parasitaemia the ticks selected had engorged on were; rising to <0.1%, <0.1 - 0.2%, 0.2 - 0.8%, 1.3 - 2.1% and falling from 2.4 - 0.8%, 0.5 - 0.1%, 0.1 - <0.1%.

The salivary glands to be analysed by PCR were removed and placed directly in 180µl of Buffer ATL (tissue lysis buffer). The ticks were stuck in wax in separate Petri dishes and a new scalpel blade was used for each dissection, to minimise the

chances of DNA cross contamination. Forceps and needles were wiped with a clean tissue, dipped in 100% ethanol and flamed after each use. The glands for MGP staining and microscopical examination were teased out on slides as described previously (Chapter 2).

3.7. *Comparing* T. parva *infection in the same tick with PCR analysis and microscopy*

Four groups of 30 ticks (15 males, 15 females) were used giving a total of 120 examined. The groups were selected on the basis of count data available from the previous experiment. The groups selected were: uninfected ticks (controls), those which detached at <0.1%, <0.1 - 0.2% and 2.4 - 0.8%. It was thought that these batches would offer a well graded range of infectivity. The ticks were processed as described above. The gland to be used for PCR was removed first (to avoid potential contamination from the other gland) and placed directly in 180µl of Buffer ATL. The glands to be stained were teased out on individually marked slides and dealt with as described previously. The same notation on the microscope slide and the PCR tube number enabled a comparison of the results of light microscopy with those of PCR.

After the salivary glands were stained, the markings on the slides were covered with sticky labels so they could not be identified. They were then thoroughly randomised and had a code from 1 - 120 assigned. A supervisor who had not previously seen the slides carried out the infection assessment. After the count data had been tabulated along with the code number, a second supervisor removed the labels and on a separate data sheet recorded the slide number with the code. The count data were matched with the slide from which it was extracted. The intensity of the PCR product band was recorded on a direct visual ranking scale from 0 (no band) to 5 (large, bright band).

3.8. *Assessing whether the tick bloodmeal inhibits PCR analysis*

Two batches of ticks were used; 60 (30 males, 30 females) from the <0.1 - 0.2% and 60 from the 1.3 - 2.1% parasitaemia. Each batch was split into two groups of 30 (15

males, 15 females), one of which was untreated and the other was partially fed on NZW rabbits for 4 days to allow their sporoblasts to mature. The body length of partially fed female ticks was measured in millimetres from the cervix to parma and recorded when they were removed from the rabbit to give an indication of the size of bloodmeal they had imbibed. The data would give an indication of which ticks had fed the most (the females had not reached Stage 3 of engorgement) to allow correlation with PCR detection of infection. All the ticks were placed in 100% ethanol and allowed to fix for a minimum of 2 days. They were bisected in individual Petri-dishes using a new scalpel blade each time and both halves placed together in 180µl of Buffer ATL. The bisection was to allow maximum exposure of tissue to the digestive enzymes. The rest of the digestion and amplification proceeded as described in the previous experiments. The amplified products were run on 1.6% agarose gels in batches of 15 for comparison between groups.

DNA was extracted from 200µl of rabbit blood and tested by the PCR before and after tick application to ensure there was no cross-reactivity with *T. parva* DNA and that *T. parva* DNA was not circulating within the bloodstream. DNA was extracted from uninfected, unfed and partially (rabbit) fed ticks and used as control material in the PCR.

All raw data was checked for normality before the appropriate statistical test was selected. The mean and standard deviation was calculated for each sample to check if 70% of the observations fell within the interval $\xi \pm s$. If this was the case, the data was considered normal and parametric tests were selected, if not, non-parametric tests were used, as the data was considered non-normally distributed.

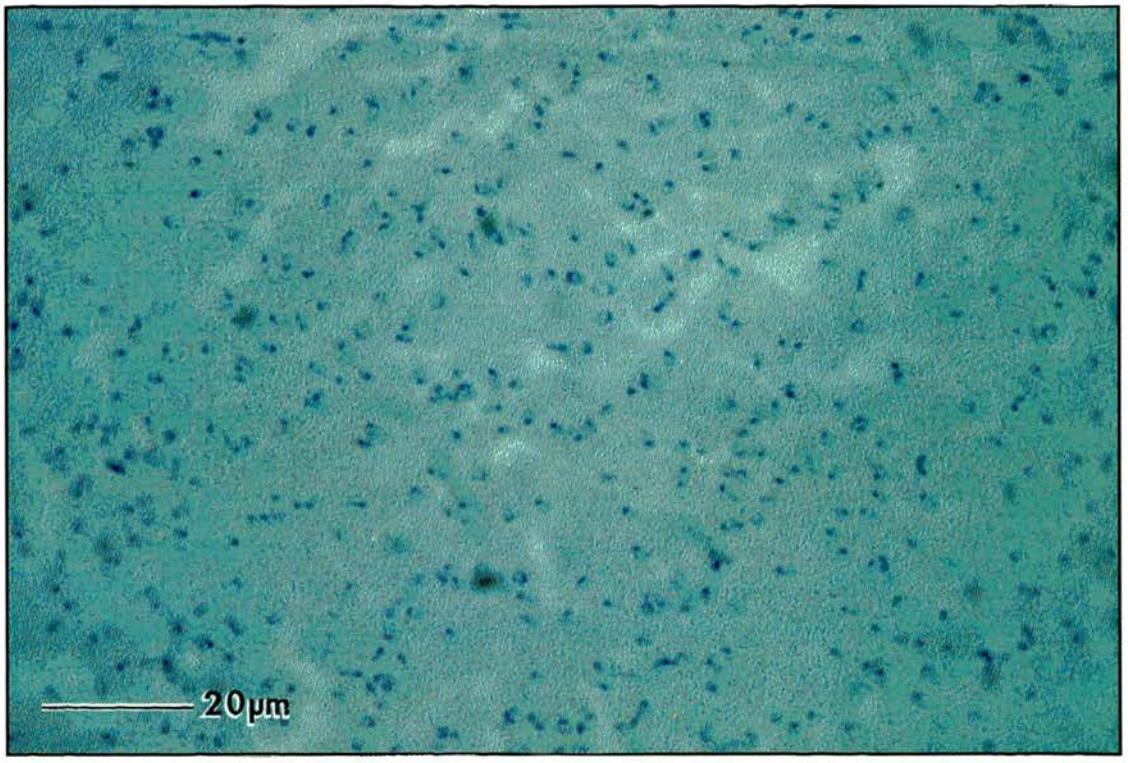
Results

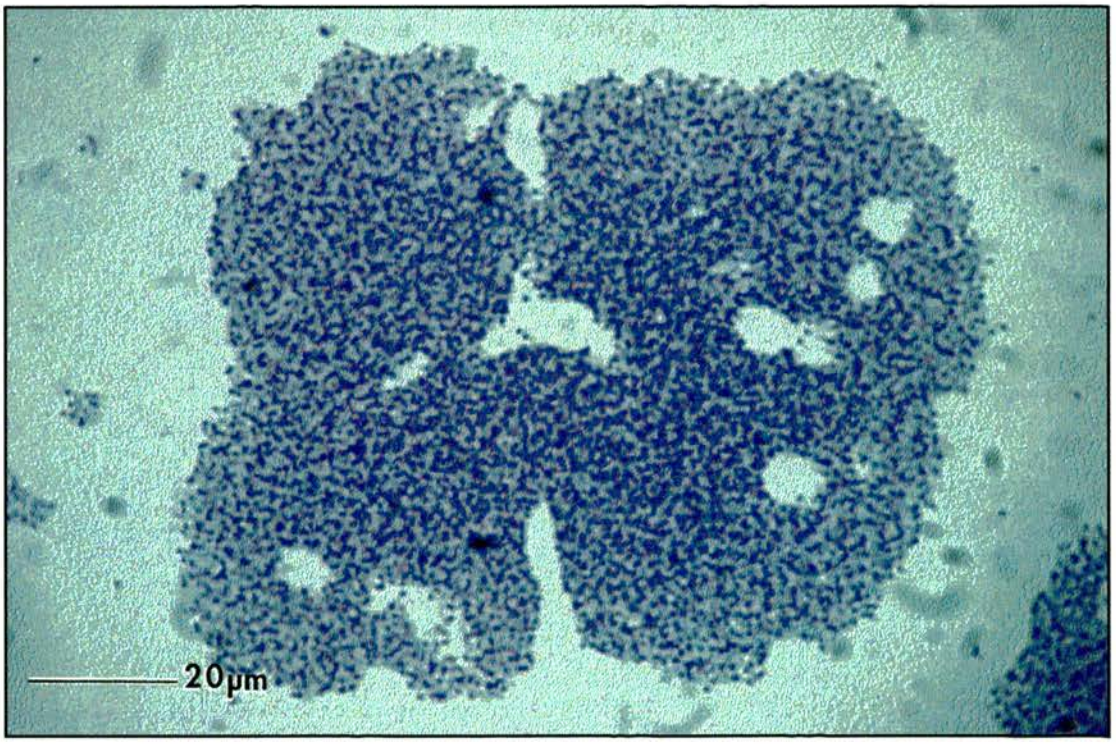
3.9. *Preparation of positive control material*

The piroplasm extraction was successful in that very large numbers of piroplasms were purified, free from contaminating bovine white blood cells. Figs. 3.1a and 3.1.b. shows cytocentrifuge preparations of the piroplasms at different points in the

Fig. 3.1a. A cytocentifuge preparation of bovine erythrocytes, many of which are infected with *T. parva* piroplasms.

Figure 3.1b. A pure, extracellular preparation of *T. parva* piroplasms. Piroplasms were usually seen in large aggregated masses like the one pictured.


20µm


20µm

extraction protocol. The extracted piroplasms were extremely sticky and tended to form very large clumps that were difficult to pipette to homogeneity.

3.10. *PCR using* T. parva *and* T. taurotragi *DNA*

Fig. 3.2. shows a representation of the gel obtained from the PCR reaction with *T. parva* and *T. taurotragi* DNA. Tick derived *T. taurotragi* produced two bands oflower molecular weight than the TPR1 amplicon at 405bp. The amplified products were not sequenced, so exact sizes cannot be given. Comparing the bands with those from DNA size markers, they are estimted to be 320 and 350 base pairs in length. The intensity of the *T. taurotragi* bands were weaker than that of *T. parva*.

3.11. *Assessing tick infections by PCR analysis and microscopy.*

Fig. 3.3 shows PCR and MGP results from the comparison of dissected glands from paired ticks. PCR appears to detect more infection than microscopy when the parasitaemia was dropping, but a two-tailed, paired t-test performed on the data revealed that there was no significant difference between the means of the data sets ($P = 0.689$) and that a significant correlation existed ($r = 0.9017$). A Wilcoxon matched-pairs signed-ranks test performed on the paired gland count data showed a non-significant difference between the infections in each gland ($P = 0.490$). Six out of 70 ticks (8.6%) contained sporoblasts in one gland and not the other. Microscopy detected 42 / 70 (60%) infections, while PCR detected 37 / 70 (53%). A statistical analysis on the results showed that any differences between the two detection methods were non-significant (Mann-Whitney U-test, 95% confidence level). The range of sporoblast numbers (assessed by microscopy) was greatest in PCR categories 4 and 5. Much less variation occurs in PCR categories 0 to 3. The raw data from the MGP stained, paired gland counts is shown in Table 1A, Appendix 1.

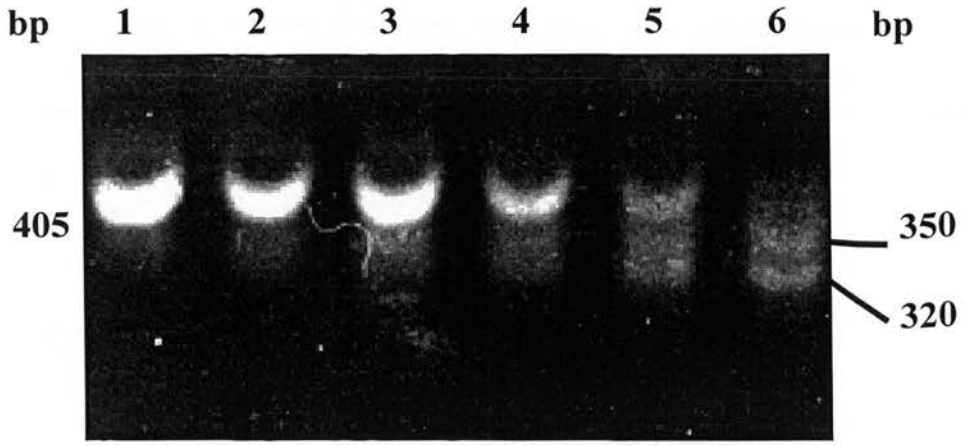
3.12. *Comparing* T. parva *infection in the same tick by PCR analysis and microscopy*

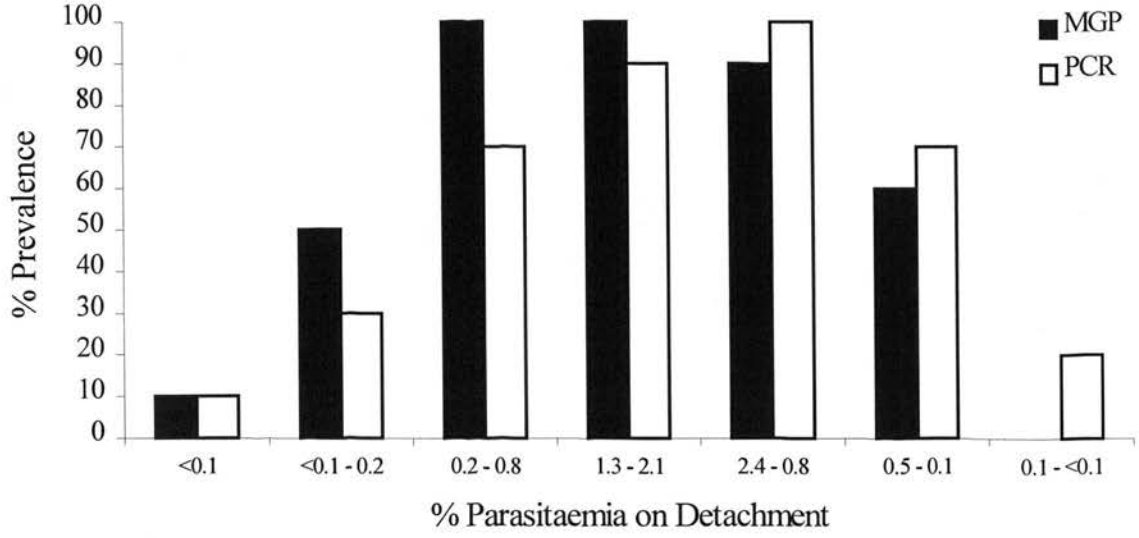
The PCR band intensities obtained allowed a relative grading on a scale of 0 (no infection detected) to 5 (high infection status) as can be seen in Fig. 3.4. Infection

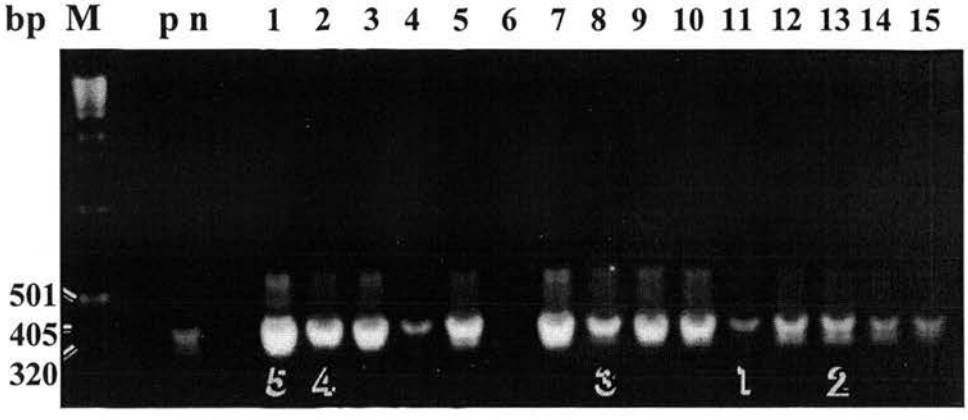
Figure 3.2. Gel comparing amplified products from *Theileria parva* and *Theileria taurotragi* after TPR1 primer PCR. DNA was extracted from *T. parva* and *T. taurotragi* infected, tick-derived stabilates. PCR was used with different ratios of *T. parva* and *T. taurotragi* DNA to establish whether they could be distinguished by their amplicon profile. The DNA was not quantified in a spectrophotometer (see text) so the volumes given are the amount of sporozoite stabilate, extracted DNA added to each 100µl PCR. [Lane] **1**: 5µl *T. parva* **2**: 4µl *T. parva* / 1µl *T. taurotragi* **3**: 3µl *T. parva* / 2µl T. taurotragi **4**: 2µl *T. parva* / 3µl *T. taurotragi* **5**: 1µl *T. parva* / 4µl *T. taurotragi* **6**: 5µl *T. taurotragi*. bp - base pair size.

Figure 3.3. Graph comparing *T. parva* detection in adult tick salivary glands by PCR and microscopy. The nymphs detached from an infected calf over a piroplasm parasitaemia ranging from <0.1 to 2.4%. After moulting to adults, a total of 140 ticks were selected from 7 nymphal detachment days (10 males, 10 females from each). PCR and microscopy were then used to assess and compare the prevalence of *T. parva* in an equal number of ticks from each batch (5 males, 5 females) giving a total of 70 ticks for each technique.

Figure 3.4. Gel showing a range of TPR1 gene amplicon sizes from *T. parva* infected adult tick salivary glands after PCR. The amplicon sizes were categorised on a scale of 0 to 5 (figures below the amplicons), in which 0 represented no band (no infection) and 1 to 5 represented bands of increasing intensity (and therefore greater infection). M - Marker, p - positive control, n - negative control (no DNA), Lanes 1 to 15 - results of PCR on individual tick salivary glands.


bp
1
2
3
4
5
6
bp
405
350
320


100
90
80
70
60
50
40
30
20
10
0
% Prevalence
MGP
PCR
<0.1
<0.1 - 0.2
0.2 - 0.8
1.3 - 2.1
2.4 - 0.8
0.5 - 0.1
0.1 - <0.1
% Parasitaemia on Detachment


bp M
p n
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
501
405
320
5 4
3
1
2

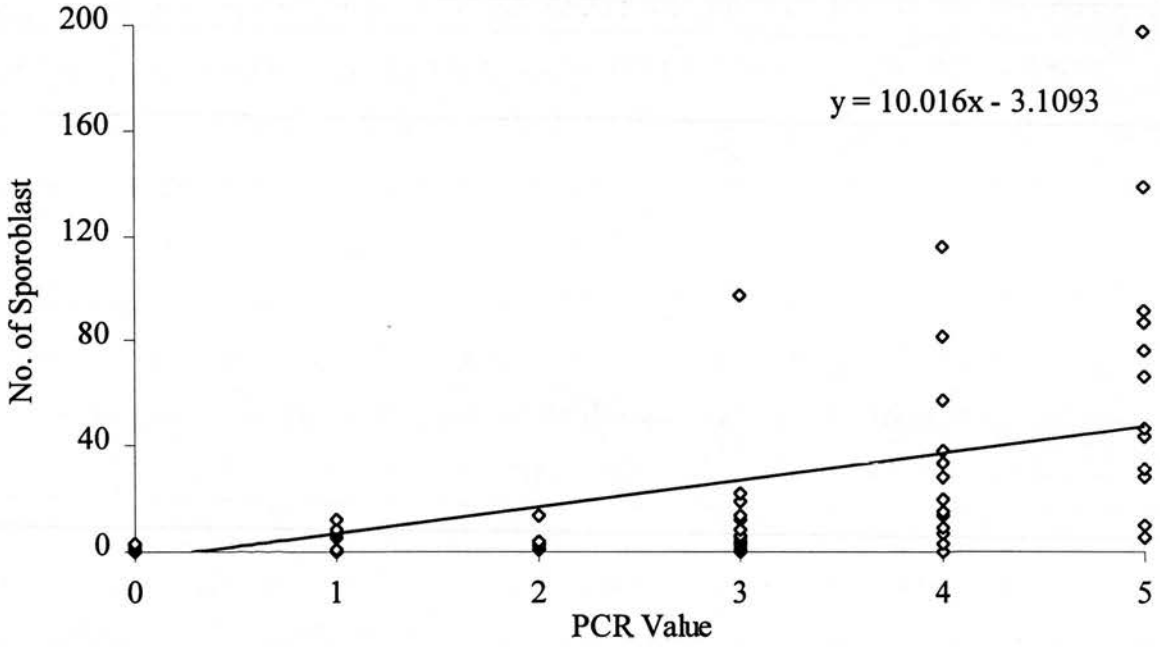
was detected in 10 out of 90 (11.1%) ticks by PCR and not microscopy and 5 out of 90 (5.6%) ticks by microscopy and not PCR, but a Wilcoxon matched-pairs signed-ranks test showed the differences to be non-significant (P = 0.277). Fig. 3.5. shows a graph correlating the sporoblast count data with the values assigned to the PCR band intensities. A Spearman Rank Correlation test gives a r value of 0.8445 (95% confidence level, P < 0.0001 showing r is significantly different to 0) shows a positive correlation between the intensity of the PCR products and the sporoblast counts. The raw data from this experiment is shown in Tables 1B + 1C, Appendix 1.

3.13. *Assessing whether the bloodmeal inhibits PCR analysis*

Table 3.1. shows PCR band intensity and prevalence figures for the two groups of ticks. As can be seen from the table, blood feeding had a significant impact on the PCR products. The size of the ticks ranged from 3.2 to 6.8mm (mean - 4.5mm, s.d - 0.6) and a Spearman's Rank Correlation showed a non-significant correlation between tick size and PCR band intensity. Partially fed ticks showed higher prevalence values than unfed ticks and the intensity of PCR products was greatly increased. The greatest effect can be seen in the results from ticks which detached at <0.1 - 0.2% parasitaemia. *T. parva* DNA was detected in 66.7% and 60% of unfed male and female ticks respectively with average group PCR band intensities of 1.47 and 1.27. *T. parva* DNA was amplified from 100% of the fed ticks and the average PCR band intensities for male and female ticks increased to 3.73 and 2.40 respectively. The prevalence figures from fed and unfed ticks from the batch that detached at 1.3 - 2.1% parasitaemia did not show the same increase as in the previous group, because the prevalence levels in the unfed ticks were already 100% in both sexes. The average group values of PCR band intensities increased upon feeding, from 2.27 to 4.73 and 2.73 to 3.8 for male and female ticks respectively. A Mann-Whitney test applied to the intensity data revealed a significant difference between fed and unfed ticks, with fed ticks showing a much higher level of *T. parva* DNA detection (P < 0.0001, two-tailed test). A Mann-Whitney test revealed a non-significant difference between average intensity levels in unfed male and female

Figure 3.5. Graph correlating the assessment of *T. parva* infection in paired tick salivary glands by PCR and microscopy. A total of 120 ticks were used, but the number of ticks represented by each PCR rank varied quite markedly. PCR 0 - 59 ticks, PCR 1 - 13 ticks, PCR 2 - 7 ticks, PCR 3 - 14 ticks, PCR 4 - 15 ticks, PCR 5 - 12 ticks. The raw data from this experiment is shown in Table 1D, Appendix 1.

Table 3.1. The prevalence and abundance of *T. parva* infection as assessed by PCR was compared in a batch of partially fed and unfed ticks that detached as nymphs at low and high piroplasm parasitaemias. Thirty ticks (15 males, 15 females) were included in each batch. Half the ticks were processed when partially fed on rabbits for 4 days to allow any sporoblast infections in their salivary glands to mature, and the other half were processed in an unfed state. † - male or female ticks, * - the average intensity of the amplicons from each tick batch. Prevalence refers to the percentage of ticks detected as being infected with *T. parva*.

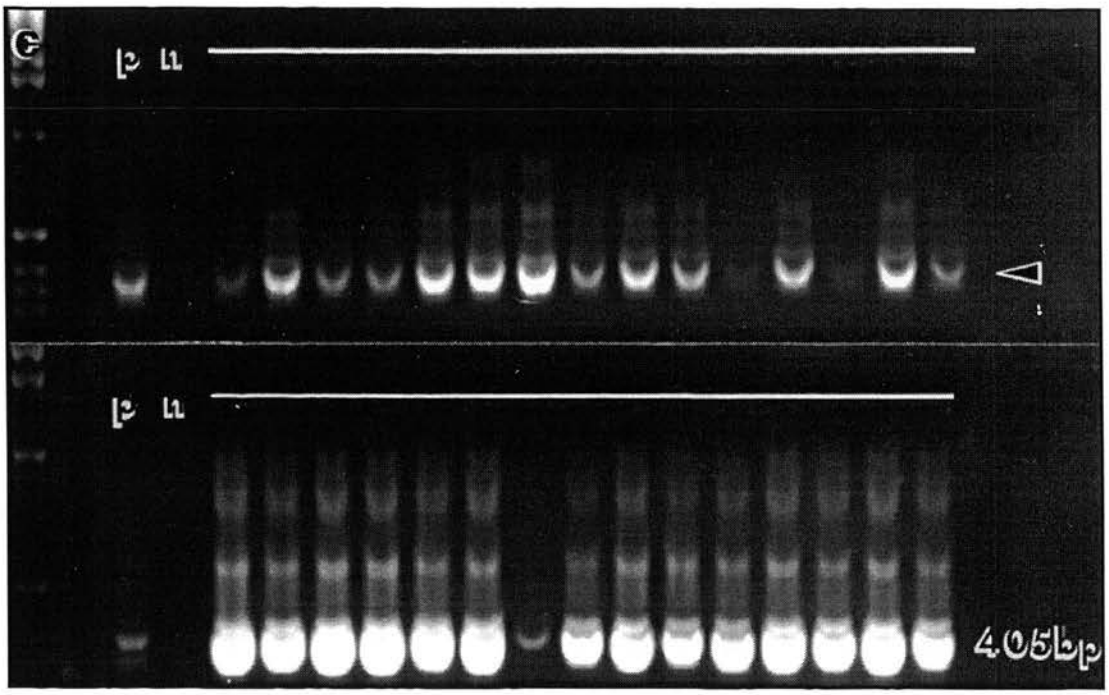


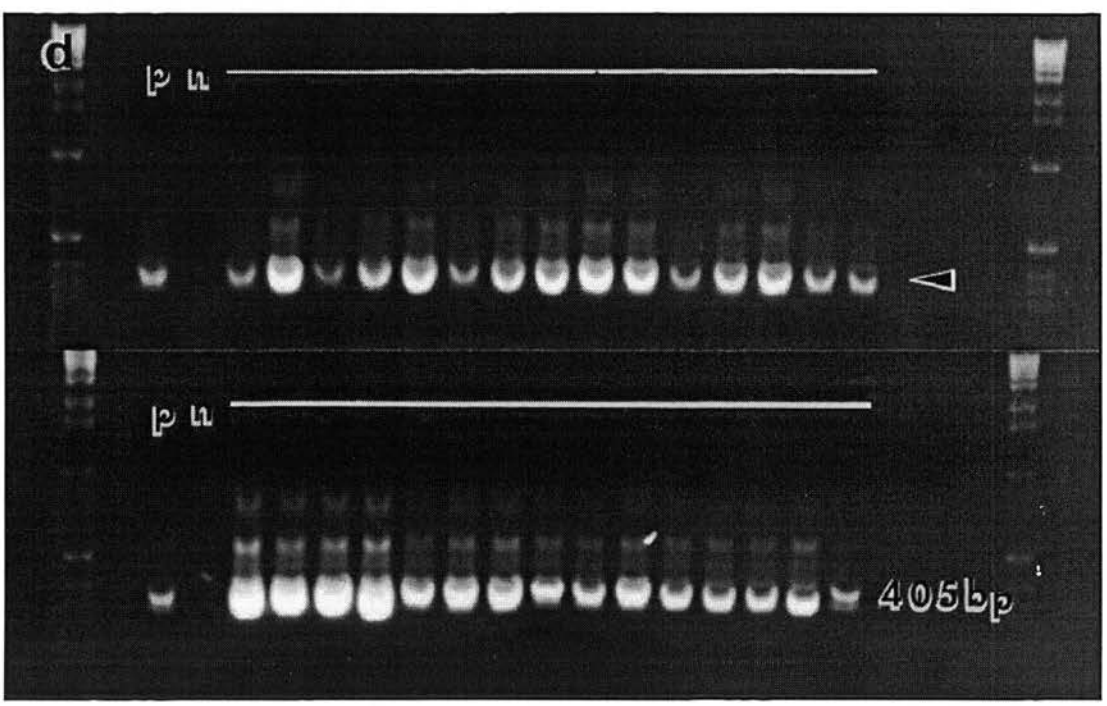
Piros (%)	m / f†	Fed / Unfed	Prevalence (%)	Av. Intens*
0.2	m	unfed	66.7	1.47
''	f	unfed	60	1.27
0.2	m	fed	100	3.73
''	f	fed	100	2.40
2.1	m	unfed	100	2.27
''	f	unfed	100	2.73
2.1	m	fed	100	4.73
''	f	fed	100	3.80

Figure 3.6a - d. Gels showing PCR detection of *T. parva* in whole, alcohol fixed, unfed and partially fed adult ticks that detached as nymphs at low and high piroplasm parasitaemias. The experimental aim was to examine the effect of bloodfeeding on the amplification reaction. Half the ticks were partially fed on rabbits for 4 days to allow any sporoblast infections to mature, while the other half were processed in an unfed state. The two batches used, detached from an infected calf as nymphs at 0.2% and 2.1% piroplasm parasitaemia. 15 male and 15 females were processed from each group. **a**. top row: 0.2% male unfed, bottom row: 0.2% male fed. **b**. top row: 0.2% female unfed, bottom row: 0.2% female fed. **c** (overleaf). top row: 2.1% unfed male unfed, bottom row: 2.1% male fed. **d** (overleaf). 2.1% female unfed, bottom row: 2.1% female fed. p - positive control, n - negative (no DNA) control. The white line spans the wells in which the 15 tick samples were loaded. The arrowhead points to the 405bp amplicons.

a
405bp

b
405bp


c
405bp


d
405bp

ticks, but a significant difference between the levels in fed male and female ticks with male ticks showing a much greater level of infection ($P < 0.01$). Fig. 3.6 shows PCR results from unfed and partially fed male and female ticks from batches that detached at 0.2 and 2.1% piroplasm parasitaemia. No amplified products were seen from DNA extracted from rabbit blood (before and after tick application), unfed or partially fed control (uninfected) ticks.

An interesting observation was seen upon dissection of partially fed ticks from the batch that detached at 1.3 - 2.1% parasitaemia. Many showed salivary glands that were bare of acini except at the distal ends of the branched ducts, very fragile guts that tore easily on removal, and haemolymph that appeared to be contaminated with gut derived material giving it a brown appearance.

Discussion

Piroplasm extraction provided a very pure and high quality source of DNA for control PCR reactions. The extracted DNA was not quantified on a regular basis with the spectrophotometer because the PCR technique used was not directly quantitative. Most of the samples dealt with in the experiments involved extracting DNA from *T. parva* infected ticks for PCR. The very large, intense bands produced from some of the samples, particularly the partially fed ticks in the final experiment showed that large quantities of *T. parva* DNA were obtained by the technique. However, even a large amount of *T. parva* DNA will be highly diluted by tick and/or rabbit DNA under these conditions. Quantifying DNA from extractions of this sort will not reveal anything about the amount of *T. parva* DNA specifically, making initial quantitation procedures unnecessary.

The process of discriminating between *T. parva* and *T. taurotragi* is made difficult at a molecular level because they share so much sequence homology (Bishop *et al* 1994). The results from these experiments show it is possible to distinguish between the two species on the basis of the band profile displayed on a gel. The intensity of the *T. taurotragi* amplicons were less than that of *T. parva*, but it is not known if this

is a result of fewer sporozoites in the *T. taurotragi* stabilate used for DNA extraction or more copies of the TPR1 region in *T. parva*, leading to higher primer annealing levels and a stronger signal. No other tick derived stabilates of *T. taurotragi* or further information on the one used were available, so it was not possible to investigate these issues.

PCR and MGP prevalences obtained from unfed tick salivary glands were very similar and showed PCR to be a viable alternative to microscopical examination. If the detection of infection in unstimulated salivary glands was the only concern, PCR may actually be preferable to microscopy as there is less scope for subjective interpretation. Because the glands are unstimulated, the sporoblasts are often very thin, crescent shaped structures around the outer edge of Type III acini. They will have very little RNA transcription occurring and so MGP will not stain very intensely, making identification somewhat difficult. PCR bands produced on the other hand were always discernible, even at the level where only one sporont may have been present.

The sporoblast counts from MGP stained, paired glands revealed a non-significant difference between the infection levels in each gland. When the infection was at a low level, it was possible to have infection in one gland and not the other, as was the case in 8.6% of the glands examined. In experiments using five *T. parva* stocks in a single tick stock, Chen *et al* (1991) found this figure to be 12.5%. This data is necessary because it gives an indication of an acceptable and/or expected discrepancy between PCR and microscopy results obtained from separate glands within individual ticks which may have low level infections *i.e.* those in the batches which detached on <0.1% or <0.1 - 0.2% parasitaemias. Although we did not specifically isolate one sporoblast in an otherwise uninfected salivary gland, PCR did prove capable of detecting parasites in samples from ticks in which only one sporoblast was observed by microscopic examination of the paired gland. Chen *et al* (1991) used DNA probes to detect *T. parva* in salivary glands and demonstrated parasites in one gland of a pair in which the other had been shown to contain one sporoblast.

As can be seen in the graph correlating the sporoblast count data with PCR band intensities, there was a large range in count data within every PCR category. Microscopical examination of MGP stained glands frequently revealed variation in the relative maturity of sporoblasts in glands from unfed and partially fed ticks. With untreated ticks however, this variation in the salivary glands tends to be more pronounced. It is common in these cases for the majority of sporoblasts to be immature, but, in a few cases, there are some that are considerably larger and semi-mature. These sporoblasts contain substantially greater amounts of *T. parva* DNA and can therefore account for PCR values that are higher than would be expected for the actual number of sporoblasts present as determined by the MGP count from the paired gland. This indicates that PCR may not be suitable for use with stimulated salivary glands if an indication of intensity of infection is required. The sporoblasts in these glands could become hugely expanded and the danger would be that a low number of sporoblasts could account for a large PCR band intensity that would not be comparable with results from unstimulated glands, even if they contained considerably larger numbers of immature sporonts. However, if a measure of prevalence is all that is required, PCR is highly suitable.

PCR used here is only quantitative on a relative scale. The end-point examination of reaction products is very likely to occur after the plateau effect has occurred. PCR bands recorded as 4 or 5 are almost certainly an example of this phenomenon. It is likely that within these amplicon size categories, there is a very large range in the number of sporoblasts. However, for the purposes of this study, the limited data provided by PCR in terms of *T. parva* abundance is sufficient. The results indicate an increase or decrease in parasite load and provide a comparison between tick groups which is more relevant to this study than accumulating data on exact sporoblast numbers.

PCR on salivary glands from partially fed ticks produced a great deal more amplified products than in unstimulated glands. No correlation was demonstrated between the size of tick (which gives an indication of the blood volume ingested) and PCR band

intensity showing that components of the bloodmeal and from the rapidly growing tick do not appear to inhibit PCR as has been shown previously (Johnson *et al* 1992).

The prevalence of *T. parva* detection in the batch of ticks that detached at <0.1 - 0.2% parasitaemia was greater in the partially fed ticks than the untreated ones. Upon feeding, most of the average PCR band values rose from 1.37 to 3.2 indicating a substantial increase in parasite DNA. 37% of unfed ticks were uninfected according to the PCR and 37% of the partially fed ticks produced PCR bands in the 1/2 category. The difference between the 1/2 and 4/5 intensity bands seen in the partially fed ticks was very stark and raised interesting questions. DNA extracted from rabbit blood, collected from a vein on the same ear the ticks were feeding, did not amplify suggesting that sporozoites were not present in the circulating blood. The ticks fed in a very tightly bunched group in the rabbit's ear and would have had their mouthparts in very close proximity to each other. The partially fed ticks that produced PCR band values of 1/2 may have actually been uninfected ticks that ingested sporozoites from infective ticks feeding beside them. This would have to be confirmed by dissecting the ticks and extracting DNA from the gut and salivary glands for a PCR to assay for the presence of *T. parva* DNA. A similar phenomenon has been demonstrated previously with the transfer of tick-borne encephalitis (TBE) virus in *R. appendiculatus* (Labuda *et al* 1993). The TBE virus can continue its lifecycle in the recipient tick by this means of transfer, but sporozoites would be unable to continue their development as they are adapted to survive in the bovine host, not the tick. The relevance of the sporozoites passing into the tick guts could be that an overestimation of infection could be generated from field collected ticks if whole ticks were processed for PCR after being removed from a crowded feeding site on a steer. One infected tick within that site could result in infection being detected in other ticks feeding in very close proximity.

The male ticks appeared to have more infection than female ticks after 4 days of feeding. The average group amplicon sizes from partially fed males was 3.7 compared to 2.4 in females in the group that detached at <0.1 - 0.2% parasitaemia

and 4.7 compared to 3.8 in females in the group that detached at 1.3 - 2.1% parasitaemia. It was shown that infection levels measured by PCR and microscopy in unfed ticks do not differ significantly between male and female ticks, so the differences seen in the partially fed ticks are unlikely to have arisen from an initial imbalance in their infections. Male ticks have been shown to feed more slowly than females and consequently, their sporoblast infections develop and mature over a longer period. By day 4 of feeding, it is likely that sporozoites will have developed and been ejected in the saliva of female ticks leading to a reduction in their parasite load and subsequent PCR band intensity.

The results showed that the TPR1 based PCR is a specific, accurate and reliable technique for detecting *T. parva* in alcohol preserved ticks. It is more suitable for use with unfed ticks (those likely to be collected from vegetation rather than an animal) because of the effect activated sporoblasts have on increasing the amplicon intensity. For studies that allow live ticks to be returned to the lab, PCR could be used in conjunction with salivary gland staining techniques because the parasite species could be identified without the need to infect susceptible animals. A proportion of the ticks could be assessed by both methods and have the sensitive detection and species-specific benefit of PCR with the ability to gather accurate abundance data from microscopy. An area where eland and cattle come into close contact could theoretically contain *R. appendiculatus* with *T. parva* and *T. taurotragi* in their salivary glands. The best protocol for confirming local infection prevalences for epidemiological purposes would be a collection of more than one hundred unfed ticks from vegetation and a 24 hour attachment period on sentinel cattle. If the ticks were returned live to the laboratory, a number of them could be dissected, their salivary glands stained and DNA could be extracted from the remainder for PCR to allow accurate species identification. If the ticks have to be preserved in ethanol, they could be processed in groups of ten and an approximate prevalence could be calculated using the formula of Chiang and Reeves (1962).

CHAPTER 4

A PCR-based field evaluation of *Theileria* infections in cattle and ticks in Kenya

A field survey was carried out on three field sites in Kenya to assess *T. parva* prevalence in cattle and ticks in relation to vaccination trials either proceeding or planned in the areas.

Introduction

The work described in this chapter was carried out in conjunction with the National Veterinary Research Centre (NVRC) in Muguga, Kenya.

Around sixty percent of the animals sampled in Limuru had been vaccinated with the *T. parva* vaccine in 1995 and 1996. Some animals would have received two immunising doses but accurate records are not available. The vaccine was *T. parva* Marikebuni GUTS stabilate 3014. An assessment of cattle and tick infection levels was desired to compare with those from before the vaccination trial and with other areas which had not been vaccinated. The areas Kitale and Kakamega were unvaccinated and were being considered as experimental areas in which the vaccine could be introduced. In these cases, checking for the pre-vaccine level of *T. parva* was essential before any decision could be made as to the suitability of the area for a test site.

Theileria velifera and *Theileria mutans* are widespread parasites of cattle and buffalo and the main tick vector is *A. variegatum*. Both species tend to be of low pathogenicity in cattle, although Young (1978) reported that some stocks of *T. mutans* from Cape buffalo did cause severe pathogenesis in cattle. The ticks were commonly found in Kitale and Kakamega making the presence of the parasites in these areas a distinct possibility. Discriminating between different *Theileria* species is very difficult at a morphological level. Norval *et al* (1992) stated that a thin blood

film is of no value as a *T. parva* diagnostic test in the absence of clinical data and lymph node biopsy, as detecting piroplasms may merely indicate the carrier status in a clinically normal animal. In all theilerial species, a variety of piroplasm forms are seen, including rod, oval, comma shapes and rings. Although the different *Theileria* species have characteristic piroplasm forms that predominate, they can vary with the course of infection, rendering them unreliable for speciation purposes. Schizonts from *T. mutans* have been clearly distinguished from those of other *Theileria* species on the basis of their larger size (Young *et al* 1978). However, *T. mutans* schizonts are very transient in their occurrence (Young 1981), that further decreases the probability of their detection and identification. Accurate data collection on *T. parva* prevalence will rely on discriminating between these species.

The NVRC wished to assess the presence of other tick-borne diseases within the areas. Because time constraints made a comprehensive search for all possible tick-borne pathogens in the area unfeasible, heartwater was selected as one of the more important diseases to assay for, especially as so many tick vectors were collected at two of the sites. If a consequence of vaccine administration was to cause immunosuppression, even for a brief period, vaccinated animals may be rendered more susceptible to a range of diseases they were not protected against. Therefore, checking for the presence of other *Theileria* sp. and tick-borne disease agents within a pre-vaccinated areas is a desirable precaution.

Heartwater (cowdriosis) is a tick-borne disease of cattle, sheep, goats and some wild ruminants and is caused by a rickettsia, *Cowdria ruminantium*. Typically, the disease is characterised by high fever, nervous signs, oedema of the lungs and brain, and death. It occurs in most African countries south of the Sahara and is one of the major causes of stock losses in southern Africa. The disease is transmitted by nymphal and adult instars of *Amblyomma* species. Unlike *T. parva*, which is cleared from the nymphal and adult salivary glands upon feeding, *Amblyomma* ticks retain their infection enabling transstadial transmission from pickup by larvae through to transmission back to livestock by adults. The occurrence of the disease is suppressed

by the use of tick control, the resistance of certain animal breeds and endemic stability.

Three field site areas were chosen for study relating to the delivery of the live *T. parva* vaccine. One of the primary reasons for selecting the three areas chosen over others was the presence of suitable nearby infrastructure in the form of Kenyan Agricultural Research Institute (KARI) field centres, or the NVRC itself. The areas were also considered to be typical in terms of tick challenge and cattle susceptibility. Fig. 4.1 shows the location of the three areas.

Figure 4.1. Map of Kenya showing the locations of the field site areas.

Limuru is located about 15 kilometres north west of Nairobi in the lower highland zone of the Central Province. The area is characterised by hills, plateaus and high level structural plains. The altitude ranges from 1500 to 1800m above sea level and the average temperatures range from 12.5^{0}C in July/August to 20.4^{0}C in March/April.

There are two rainy seasons; the long rains occurring from April to May and the short rains from October to November with an average of 900mm rainfall each year. High rainfall is consistent over the years and as a result, the area is highly productive with arable crops and dairy livestock. The farms visited for this study were all small-holder dairy farms. The cattle were kept in zero grazing stalls and fodder was cut from the surrounding area and brought to them. The cattle in this area were predominantly Grade cattle - indigenous zebu crossed with exotic breeds, mainly west European taurine cattle. The cattle are currently about 15 to 16 generations from the East African Short Horn Zebu and resemble black and white Holstein/Friesian breeds, but Jersey, Guernsey and Ayrshire lines are also present. The area is endemic for ECf and tick control has to be practised, although acaricide application tended to be rather sporadic and varied greatly from farm to farm.

Kakamega is situated in the Western Province near the Lake Victoria basin. It ranges in altitude from 1250 to 2000m (classed as Lower Midlands 1 - 3) above sea level and the average rainfall varies from 1000 to 2400mm at higher altitudes. The temperature means range from a minimum of 11 to 16^{0}C to a maximum of 26 to 32^{0}C and the area is very humid because of proximity to Lake Victoria which provides rain all year round with peaks in May and August. The district is characterised by a low rate of urbanisation and around 76% of the total area is under cultivation and livestock holding. Relatively fertile soils allow families to subsist on small parcels of land where they keep a small number of cattle. The farms visited for the purposes of this study stocked only indigenous, unimproved *Bos indicus*, East African Shorthorn Zebu cattle. The cattle were free to graze around the farm paddock, but the limited vegetation meant that fodder was cut from outwith the farm and brought to the cattle. No form of tick control was practised by the farmers in this area. Kakamega Forest is closely located to the study area, but presence of wildlife around the farms is rare, although waterbuck have been reported. Because the area is warm and humid, *Rhipicephalus appendiculatus* and *Amblyomma variegatum* can be found all year round on animals and the vegetation.

Kitale is located about eighty kilometres north of Kakamega, just east of Mount Elgon on the Kenyan/Ugandan border. The area is higher in altitude at 2000 to 3000m (classed as Lower Highlands 1 - 3) above sea level and consequently has significantly reduced average temperatures. Peak rainfall (average of 150mm) occurs in May and the average annual rainfall is about 1200mm. Earlier this century, most of the Kitale area was developed by European settlers into large commercial farms, in stark contrast to the small scale, subsistence farms seen in Kakamega. However, the farms sampled in the course of these experiments were small holder dairy farms. The area, in common with Limuru, is stocked mainly with exotic breeds, but in this case, the cattle were allowed to graze freely on the paddock on most of the farms we visited, but fodder had to be gathered from elsewhere. The low temperatures and low rainfall (relative to Kakamega) make this area marginal for tick survival. They are only found questing between October to April and even in this period, their numbers are very low without any form of control measures.

Because Kitale and Kakamega were being considered as field trial sites for the live *T. parva* sporozoite vaccine, it was essential first to assess the prevalence of *Theileria* infections within the areas. This is important, primarily because the likely challenge risk has to be established before the appropriate immunisation stocks or drug delivery systems are chosen. Norval *et al* (1992) stated that before large scale immunisation projects are undertaken in the field, it is essential to establish the immunising dose of sporozoite stabilate of the *T. parva* stock(s) that will not overcome the oxytetracycline regimen chosen. Limuru had been vaccinated with live vaccine on two occasions previous to our sampling period. Delivering the vaccine to an area is likely to increase the number of carrier animals present (Mutugi *et al* 1988), which in turn may have a significant effect on transmission dynamics and endemic stability. Examining the infection level in cattle and ticks was therefore essential in monitoring the ecological effects of vaccine administration.

Materials and methods

Blood samples were taken from cattle and assessed for *Theileria* infections by examining Giemsa's stained blood smears and PCR analysis. Ticks were removed from cattle after whole body counts and were assessed for infection by PCR analysis.

4.1. *Collection of samples*

4.1.a. Blood Samples

10ml blood samples were collected in vacutainer tubes that contained approximately 1ml of acid citrate dextrose (ACD) anticoagulent (see Appendix 6). Jugular vein samples were taken preferentially, but if animals were difficult to handle, their movements were restricted in a crush and the samples obtained from the tail vein. The tubes were stored in wet sand for a maximum of 2 days until they were returned to the laboratories at the NVRC. DNA was extracted from 200µl volumes using the QIAamp Tissue extraction kit. Blood smears were prepared by fixing the smear in 100% methanol for 3 min and placing them in 5% Giemsa stain for 40 mins. They were then gently rinsed with Giemsa buffer and after drying, examined under a light microscope (x100 objective).

4.1.b. Tick Samples

Animals were examined over both sides of their body for the presence of ticks. Ticks were removed and placed in tubes which were then sealed with a cotton wool bung and stored in wet sand until they were returned live to the laboratory. At the laboratory they were identified and split into different groups depending on species, sex and their physical condition. Semi-engorged ticks were stored at 18^0C, 85% relative humidity until their salivary glands were dissected out. Live-unfed ticks, removed from cattle were placed in rabbit ear bags and allowed to feed for 4 days before being removed. This allowed any potential salivary gland infection to mature, after which their salivary glands were dissected and analysed by PCR. Some of the tick guts from ticks that had partially fed on cattle were removed at the same time as the salivary glands and processed for PCR analysis. Dead ticks were placed in 100% ethanol and stored until there was time to process them. Tick material from which

DNA was to be extracted was placed in 180µl of tissue lysis buffer and the procedure followed was according to manufacturer's recommendations (QIAgen). Dead ticks were always bisected to allow maximum exposure of their viscera to Proteinase K and lysis buffer prior to DNA extraction.

Blood samples were examined by microscopy and PCR analysis to increase the chance of parasite detection. The choice of primers used on samples collected from each study site were determined by the tick species found in those areas. *R. appendiculatus*, known to transmit *T. parva* and *T. taurotragi*, were tested using the TPR1 primers (Bishop *et al* 1992) which are specific for those species. *A. variegatum*, known to transmit *T. mutans* and *C. ruminantium* were tested with *Theileria* genus primers (Allsopp *et al* 1993) and *C. ruminantium* primers (unpublished data, K. Sumption; CTVM, personal communication). Since *R. appendiculatus* was found at each field site, the TPR1 primers were used on samples from the three areas. *A variegatum* was found only in Kitale and Kakamega, which meant the use of *C. ruminantium* primers were restricted to these areas.

Fifty nine blood samples were collected, and all were examined by bloodsmear and/or PCR analysis. A total of 1026 *R. appendiculatus*, *A. variegatum* and *B. microplus* ticks were collected and 554 (54%) were analysed by PCR.

4.2. Testing primers and PCR

Information on the primers used and the expected PCR products is shown in Table 4.1.

4.2.a. *Cowdria ruminantium* PCR

Five *Cowdria* primers were designed from *C. ruminantium* Welgevonden strain DNA sequence data available on the Daresbury Database.

Table 4.1. PCR primer information for *Theileria* sp., *T. parva* and *C. ruminantium*. TPR1 primers are specific for *T. parva* and *T. taurotragi*, primers 989/990 are more general *Theileria* sp. specific and the GroE primers specifically amplify *C. ruminantium*. Although, five *C. ruminantium* GroE operon primers are displayed, the two in bold type were selected for continued use. size (bp) refers to the size of amplicon produced with the primer combinations. † refers to the size of the amplicon produced using the two primers in bold type.

Table 4.2. Specificity of the *T. parva*, *Theileria* sp. and *C. ruminantium* primer sets. * - *T. parva* TPR1 primers, † - *Theileria* sp. primers, ‡ *C. ruminantium* primers.

Primer (bp)		Nucleotide Sequence: 5' - 3'	Size (bp)
C. ruminantium - GroE operon			
GroESF1	**20**	**GCTGTTGGTCCAGGGTCTTA**	**617bp†**
GroESF2	22	TACATAGTTATGAAGGAGAGTG	
GroELF	22	ATGGCAAATATGGTTGTAACAG	
GroELR1	21	TAACACTTCACGCTTCATGCA	
GroELR2	**20**	**AGTACACCTTCCCTAACACA**	
Theileria sp. - SSUrRNA gene			
989	17	AGTTTCTGACCTATCAG	1.09 Kb
990	18	TTGCCTTAAACTTCCTTG	
T. parva - TPR1 gene			
IL194	32	ATATATCCAGCCATAGCTCCTGGAATGATTGT	402bp -
IL197	32	TACAGCCAATGAGATCTCATGACACATATAGA	405bp

Primers	Species/Stocks Amplified	Species/Stocks not amplified
IL194* + IL197	*T. p* Muguga, *T. p* Marikebuni *T. p* Lanet , *T. p* Lawrencei *T. p* (buffalo derived), *T. p* Boleni, *T. taurotragi*	*T. annulata*, *T. hirci*, *T. buffeli*, *T. mutans*, *B. bigemina*, *C. ruminantium*, *E. chaffeensis*, *R. appendiculatus*, *A. variegatum*, Bovine DNA
989/990†	*T. annulata*, *T. buffeli*, *T. hirci* *T. taurotragi*	*T. p* Muguga, *T. p* Marikebuni, *T. p* Lanet *T. p* Lawrencei, *T. mutans*, *B. bigemina*, *C. ruminantium*, *E. chaffeensis*, *R. appendiculatus*. *A. variegatum*, Bovine DNA
GroESF1‡ + GroELR2	*C. ruminantium* (Welgevonden)	*T. p* Muguga, *T. p* Marikebuni, *T. p* Lanet *T. p* Lawrencei, *T. mutans*, *B. bigemina*, *E. chaffeensis*, *R.appendiculatus*, *A. variegatum*, Bovine DNA

C. ruminantium GroE operon PCR conditions

50µl reactions contained 2mM Tris-HCl, pH 8.55, 5mM $(NH_4)_2$ SO_4, 100µM EDTA, 1mM 2-mercaptoethanol, 0.05% Thesit™, 5% glycerol, 200µM each dNTP, 0.2µM each primer, *Taq* DNA polymerase (1.5u/µl). 2.5µl of DNA solution was added per reaction.

Reactions were overlaid with 50µl of light mineral oil to prevent evaporation and denatured at 95°C for 2 mins. The mixture was subjected to 30 cycles of denaturation (95°C - 1min), annealing (60°C - 1min) and extension (72°C - 1min). This was followed by a final extension cycle at 72°C for 10mins.

Testing the *C. ruminantium* primers

DNA extracted from cultured *C. ruminantium* Welgevonden elementary bodies was used to test the PCR in order to select the best reaction conditions. Reactions were varied by using different combinations of the five GroE operon primers shown in Table 4.1. with 175ng and 87.5ng of *C. ruminantium* DNA per reaction.

The specificity of the primers was assessed using DNA from a variety of other organisms that may have confounded PCR. Samples included, *Ehrlichia chaffeensis*, *Cytocoetes microti*, *T. parva* Muguga, Marikebuni and Lanet, *R. appendiculatus* and *A . variegatum*. Dilutions of *C. ruminantium* DNA from 175ng to 175pg were added to the PCR alongside the same dilutions of *E. chaffeensis* DNA. For information on the origin of all the material used for DNA extractions in this chapter, see Appendix 5.

4.2.b. *Theileria* sp. SSUrRNA gene PCR conditions

50µl reactions contained 2mM Tris-HCl, pH 8.55, 5mM $(NH_4)_2$ SO_4, 100µM EDTA, 1mM 2-mercaptoethanol, 0.05% Thesit™, 5% glycerol, 200µM each dNTP, 1µM each primer, 2.5mM $MgCl_2$, *Taq* DNA polymerase (1.25u/µl). 2.5µl of DNA solution was added per reaction. Reactions were overlaid with 50µl of light mineral oil and denatured at 95°C for 2 mins. The mixture was subjected to 30 cycles of denaturation

(95°C - 1min), annealing (55°C - 2.5mins) and extension (72°C - 2mins). This was followed by a final extension cycle at 72°C for 10mins.

Testing the *Theileria* sp. primers

The specificity of the primers was assessed using DNA from many different *Theileria* species and strains of DNA extracted from ticks known to be vectors for *Theileria* species. The DNA samples used can be seen in Table 4.2.

4.2.c. *T. parva* TPR1 gene PCR conditions

50µl reactions contained 2mM Tris-HCl, pH 8.55, 5mM $(NH_4)_2$ SO_4, 100µM EDTA, 1mM 2-mercaptoethanol, 0.05% Thesit™, 5% glycerol (contained within Thermometric Reaction Buffer), 200µM each dNTP, 1µM each primer, 2.5mM $MgCl_2$, *Taq* DNA polymerase (1.25u/µl). 2.5µl of DNA solution was added per reaction. Reactions were overlaid with 50µl of light mineral oil (Sigma) and denatured at 95°C for 2 mins. The mixture was subjected to 30 cycles of denaturation (95°C - 1min), annealing (60°C - 1min) and extension (72°C - 1min). This was followed by a final extension cycle at 72°C for 10mins.

The specificity of the TPR1 primer PCR was tested with the same *Theileria* species and strains as the PCR using 989/990. The DNA samples used can be seen in Table 4.2.

Results

4.3. *Collection of samples*

R. appendiculatus ticks were found concentrated around animals' head and ears, *R. evertsi* were collected solely from the bare skin around the anus. *A. variegatum* were concentrated around the udders and genitalia whereas *B. decoloratus* tended to be much more generally distributed around the midriff of the cattle.

The number of blood and tick samples collected from each area is displayed in summarised form in Table 4.3. The raw data from the samples collected can be seen in Table 2A, Appendix 2.

4.3.a. *Cowdria ruminantium* PCR

Fig. 4.2.a shows a gel with the various reaction products displayed. The amplicons obtained were all of the expected size. The primer combination of GroESF1 and GroELR1 (highlighted in Table 4.1) repeatably gave the strongest bands and so these primers were selected for use in the rest of the experiments.

Testing the specificity of the primers

All of the primer combinations amplified *C. ruminantium* DNA and none of the other DNA species tried. Table 4.2 shows the list of species tried in the reactions. Fig. 4.2.b. shows PCR results from a dilution of *C. ruminantium* DNA alongside equivalent dilutions of *E. chaffeensis* DNA and *T. parva*. Amplified products from *C. ruminantium* DNA were visible at the lowest dilution tried (175pg DNA in a 50µl reaction).

4.3.b. PCR with *Theileria* sp. primers

The number of species amplified by primers 989/990 are displayed in Table 4.2. The primers amplified a very limited number of species compared to the range of species previously reported. Only *T. annulata*, *T. buffeli*, *T. taurotragi* and *T. hirci* amplified. No *T. parva* Muguga, Marikebuni or Lanet cell culture, blood or tick infected samples or *T. mutans* amplified using these primers.

4.3.c. PCR with *T. parva* primers

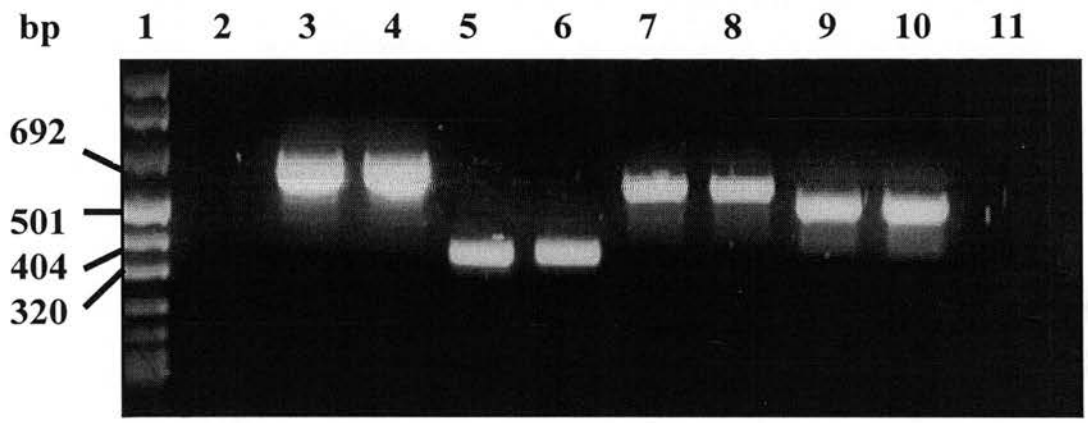
Table 4.2 shows the species specific nature of the TPR1 primer PCR with the samples tried. *T. parva* and *T. taurotragi* are the only species amplified. The combination of PCR reactions make the detection of *T. parva* (TPR1 primers), *T. taurotragi* (TPR1 + 989/990 primers), *T. annulata*, *T. hirci*, *T. buffeli* (989/990 primers) and *C. ruminantium* Welgevonden (GroE primers) possible.

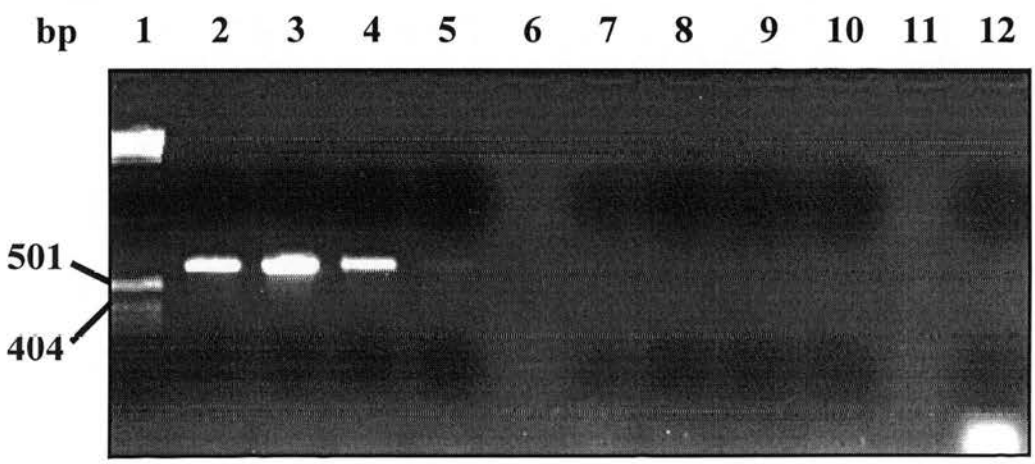
Table 4.3. The number of blood and tick samples collected and processed from the three field sites. The numbers in brackets represent the number of samples processed for PCR out of the total collected. *R. app - Rhipicephalus appendiculatus*, *R. eve - Rhipicephalus evertsi*, *B. dec - Boophilus decoloratus*, *A. var - Amblyomma variegatum*.

Figure 4.2.a. Gel showing the results from varying primer concentrations and DNA template combinations in PCR to amplify *Cowdria ruminantium* Welgevonden strain. 10µl of product were added to each well. [Lane] **1** - 1Kb marker, **2** - GroESF1 + GroELR2. -ve control (no DNA), **3** - GroESF1 + GroELR2*. 175ng *C.r* DNA., **4** - GroESF1 + GroELR2. 87.5ng *C.r* DNA, **5** - GroELF + GroELR2†. 175ng *C.r* DNA., **6** - GroELF + GroELR2. 87.5ng *C.r* DNA, **7** - GroESF2 + GroELR1‡. 175ng *C.r* DNA., **8** - GroESF2 + GroELR1. 87.5ng *C.r* DNA, **9** - GroESF2 + GroELR2§. 175ng *C.r* DNA., **10** - GroESF2 + GroELR2. 87.5ng *C.r* DNA, **11** - GroESF2 + GroELR2. -ve control (no DNA). Exp size - refers to expected amplicon size as caculated from the sequence data. * Exp size - 617bp., † - Exp size - 354bp., ‡ - Exp size - 555bp., § - Exp size - 498bp. *C.r- Cowdria ruminantium.*

Figure 4.2.b. Gel showing the results of varying the PCR conditions using primers GroELR2 and GroESF1 by adding different concentrations of *Cowdria ruminantium* Welgevonden DNA. The specificity was also tested using *T. parva* (*T.p*) and *Ehrlichia chaffeensis* (*E.c*) DNA. **1** - 1Kb marker, **2** - 175ng *C.r* DNA, **3** - 17.5ng *C.r* DNA, **4** - 1.75ng *C.r* DNA, **5** - 175pg *C.r* DNA, **6** - n.s, **7** - 175ng *E.c* DNA, **8** - 17.5ng *E.c* DNA, **9** - 1.75ng *E.c* DNA, **10** - 175pg *E.c* DNA, **11** - n.s, **12** - 175ng *T.p* DNA. n.s - no sample. *C.r – Cowdria ruminantium.*

Collection area	No. blood samples	No. ticks collected	No. *R. app* collected	No. *R. eve* collected	No. *B. dec* collected	No. *A. var* collected
Limuru	35	652 (382)	515 (306)	60 (40)	77 (36)	-
Kitale	6	91 (46)	42 (26)	10 (10)	21	18 (10)
Kakamega	18	284 (126)	132 (67)	2 (2)	28	122 (57)





DNA extracted from the *T. mutans* stabilate did not amplify with any of the *Theileria* primers.

4.4. Infection assessment

No *C. ruminantium* was detected in any cattle blood or tick sample collected from any of the field sites.

The results from *Theileria* detection in blood and ticks are displayed in Tables 4.4a and 4.4b. Table 4.5 shows the correlation between PCR and bloodsmear detection of parasites. In Limuru, PCR detected infection in 13% of samples in which microscopy did not, and microscopical examination detected piroplasms in 16% of samples in which PCR did not. A Spearman Rank Correlation showed that detection of infection by the two methods was not significantly correlated ($P > 0.05$). There was complete agreement between detection of infection with the two techniques in blood samples from Kitale and Kakamega.

A 3.2Kb amplicon was produced as a result of amplification with the TPR1 primers from most of the blood samples from Kitale and all of the samples from Kakamega. The reported amplicon size for *T. parva* with the TPR1 primers is 402 to 405bp. None of the samples processed for the TPR1 PCR in the CTVM have produced amplified products of this size. Piroplasms were seen in the related blood smears in all of the samples that showed the 3.2Kb band. All of the TPR1 primer amplified products from ticks were around 400bp in size, no tick sample produced a 3.2Kb band. Table 4.6 lists the different primer results for each *Theileria* positive tick and the blood from the animal the ticks were attached to on collection. *T. parva* amplification took place from dissected salivary glands from three *R. appendiculatus* (code: K10/2 RAf, K11/4 RAm and THI58/1 RAf). *Theileria* sp. amplification took place from dissected salivary glands of two *R. appendiculatus* ticks (code: F2A2 RAm and F2A2 RAf) and two *Amblyomma* ticks (code: F2A3 AMf1 and F2A3 AMf2). *T. parva* and *Theileria* sp. amplification took place from dissected salivary glands of a *R. appendiculatus* tick (code: F2A4 RAf). *T. parva* was detected

Table 4.4a. The number of *T. parva* and/or *Theileria* sp. detected in cattle from the three field sites by PCR analysis and microscopic examination of Giemsa stained bloodsmears. ¢ represents the collective total of *T. parva* and *Theileria* sp. detected in the samples. * - represents only those species amplified by primers 989/990. n.d - not done.

Table 4.4b. The number of ticks detected with *T. parva* and/or *Theileria* sp. infection from the three field sites by PCR analysis. ¢ represents the collective total of *T. parva* and *Theileria* sp. detected in the samples. * - represents only those species amplified by primers 989/990. '1' - may represent a false positive (see text for details). n.d - not done, n.a - not applicable.

Table 4.5. *T. parva* detection in cattle blood was compared using PCR analysis on whole blood and microscopical examination of Giemsa stained blood smears. n - number of samples on which both techniques were used. b/s - bloodsmear. The figures in brackets represent the percentage of the total numbers of samples on which both techniques were used.

Sampling Area	No.Blood	No. with *Theileria ¢*	No. with *T. parva*	No. with *Theileria* sp.*	*T. parva* and *Theileria* sp.
Limuru	35	16 (46%)	16 (46%)	n.d	-
Kitale	6	6 (100%)	2 (33%)	6 (100%)	2 (33%)
Kakamega	18	18 (100%)	-	18 (100%)	-

Sampling Area	No. *R. app / A. var*	No. with *Theileria ¢*	No. with *T. parva*	No. with *Theileria* sp.*	*T. parva* and *Theileria* sp.
Limuru	306	5 (1.6%)	5 (1.6%)	n.d	n.d
	-	n.a	n.a	n.a	n.a
Kitale	26	5 (19%)	2 (8%)	2 (8%)	1 (4%)
	10	2 (20%)	n.d	2 (20%)	n.d
Kakamega	67	'1' (1.5%)	-	'1' (1.5%)	-
	57	-	n.d	-	

Collection Area	n	Agree (%)	PCR+ve / b/s-ve	b/s+ve / PCR-ve
Limuru	31	22 (71)	4 (13)	5 (16)
Kitale	6	6 (100)	-	-
Kakamega	18	18 (100)	-	-

in DNA extracted from a pooled sample of three dead *R. appendiculatus* (code: THI36/2 RAm). *T. parva* was not detected in the host blood (showing the positive signal did not come from the bloodmeal), thereby suggesting a salivary gland infection in one of the ticks (the amplicon was very faint suggesting a low level infection in one tick). *T. parva* was detected in a dead *R. appendiculatus* tick (code: THI36/4 RAf), but it died after it had been partially fed on a rabbit for 4 days, suggesting a salivary gland infection (as any infected bovine blood present in the gut would be very unlikely to have remained after that period). Two *R. appendiculatus* ticks (code: F2A1 RAm and F2A1 RAf) were dead on processing and were both *T. parva* positive. A 3.2Kb amplicon (from the TPR1 primers) and a 1.09Kb amplicon (from amplification with the *Theileria* sp. primers) was produced from the bovine blood of the ticks' host. Only the 405bp amplicon (from reaction with the TPR1 primers) was seen in the ticks. Neither the 3.2Kb band or the 1.09Kb band were seen, showing that the *T. parva* detected in the ticks were separate infections from those found in their host, and therefore must have been present in the ticks' salivary glands. A very faint *Theileria* sp. amplicon was seen in one dead *R. appendiculatus* tick from Kakamega (code: F11 RAm). In this case the host blood was also positive for *Theileria* sp. and the chance that the bloodmeal produced a false positive cannot be ruled out. Two groups of pooled (x2 and x3), highly engorged *B. microplus* ticks (code: THI36/4 BOf1 and THI36/4 BOf2) were positive for *T. parva*. The fact that the ticks were highly engorged on *T. parva* infected blood, and that *B. microplus* ticks are known not to transmit *T. parva* shows that the bloodmeal was most probably responsible for the positive result. The amplified products from the *Boophilus* ticks were very faint, much more so than would have been expected from the volume of infected blood (which on its own produced an amplicon of medium intensity) and therefore parasites that would have been within their guts.

Table 4.6. Tick samples from the three sites in which *Theileria* infections were by found PCR analysis. s/g - salivary glands, d - dead tick, RA - *Rhipicephalus appenduculatus*, AM - *Amblyomma variegatum*, BO - *Boophilus decoloratus*, m - male, f - female. Figures in brackets represent the number of ticks that have been pooled. The letter and number series before each tick is a code referring to the farm and animal the ticks were removed from. '+' - may represent a false positive (see text for details).

KITALE		Tick		Blood	
Tick code	s/g or d	TPR1	989/990	TPR1	989/990
F2A1 RAm	d	+	-	+ (3.2Kb)	+
F2A1 RAf	d	+	-	+ (3.2Kb)	+
F2A2 RAm	sg	-	+		
F2A2 RAf	sg	-	+		
F2A4 RAf	sg	+	+		
F2A3 AMf1	sg	n.d	+		
F2A3 AMf2	sg	n.d	+		
KAKAMEGA		Tick		Blood	
F11 RAm	d	-	'+'	+ (3.2Kb)	+
LIMURU		Tick		Blood	
K10/ 2 RAf	sg	+	n.d		
K11/4 RAm	sg	+	n.d		
THI36/2 RAm	d (x3)	+	n.d	-	n.d
THI36/4 RAf	d	+	n.d	+	n.d
THI58/1 RAf	sg	+	n.d		
THI36/4 BOf1	d (x2)	+	n.d	+	n.d
THI36/4 BOf2	d (x3)	+	n.d	+	n.d

Some of the guts from *R. appendiculatus* ticks collected from the Limuru farm KANY 9 were removed and processed for PCR. The host blood showed a medium strength *T. parva* amplicon, but no amplification took place from the guts even though they clearly contained a bloodmeal. The raw data from processing the blood and tick samples can be seen in Tables 2B to 2E, Appendix 2.

Discussion

The *Cowdria* primers used were very specific for the *C. ruminantium* Welgevonden strain, from which the primers were designed. However, it has to be borne in mind that they were only tested with that one strain. Peter *et al* (1995) showed a very sensitive and specific *C. ruminantium* PCR for detecting the parasite in ticks. They

were able to detect DNA from only 10 rickettsial bodies in a tick. Quantification of the level of detection in the experiments described here is difficult to put into context because a pre-extracted DNA sample was received. The fact that DNA was extracted from elementary bodies grown in 50ml of culture does not allow an extrapolation to the DNA solution because the QIAgen filter column used to extract DNA may have been saturated with material, thereby making the DNA concentration in the eluate unrepresentative of that in the culture.

No *C. ruminantium* was detected in any of the blood or tick samples from Kitale and Kakamega. The sample size was inadequate to declare the areas free of *C. ruminantium* however. It is possible that low parasite prevalence and abundance within cattle and ticks may have gone undetected by PCR analysis. However, a number of epidemiological studies have been carried out in *C. ruminantium* endemic areas and have detected high levels within these zones. Faraougou *et al* (1998) used PCR on *A. variegatum* ticks collected in Benin and found tick infection rates ranging from 5.7 to 10%. Other studies on *A. hebraeum* in Zimbabwe showed prevalence of *C. ruminantium* of up to 36% in female ticks and 45% male ticks (Norval *et al* 1990). *C. ruminantium* antibody detection methods in cattle showed very high levels of 73 to 94% in the Kenyan Coast Province (Deem *et al* 1993) and 61 to 67% in northern Cameroon (Awa 1997).

The primers ideally should have been tested on more *Cowdria* strains than Welgevonden. Welgevonden was isolated in South Africa and it is possible that the genomes of the Kenyan parasites are sufficiently different from those of South African strains to make primer annealing unlikely. Watson 1993 (MSc Thesis, CTVM) found the primers used to amplify the Welgevonden strain did not amplify the Ball3 strain. Both these strains were isolated from the northern Transvaal, S. Africa showing genotypic differences present within a relatively small geographic area. Testing local Kenyan parasite isolates with PCR analysis would have to be carried out before any firm conclusions could be drawn from studies like the one

described. The farmers in the areas sampled were asked about disease incidence and did not report any cases of heartwater outbreaks.

Primers 989/990 did not amplify the number of *Theileria* species expected from previous publications (Allsopp *et al* 1993). Although this was disappointing, it was felt that a combination of these and the TPR1 primers would be capable of detecting a wide range of *Theileria* species. It was also anticipated that the primers would have the potential to detect *Theileria* species that had not been previously characterised.

The piroplasm levels in Limuru were very low, mostly all below 0.1%. The difference between PCR and bloodsmear detection of infection was large enough to show a non- significant correlation between the two methods in this area. The combination of bloodsmear examination and PCR analysis appeared to be an effective way of detecting the maximum number of positive samples. There was complete agreement in parasite detection between the two techniques in Kitale and Kakamega, presumably because the levels of parasites were greater. Less than 0.1% is the lowest parasitaemia value recorded in the smear assessment procedure, but within that value there can be a large range in piroplasm numbers.

One of the reported consequences of *T. parva* Marikebuni 3014 vaccination is the establishment of a carrier-state infection. Norval *et al* (1992) defined the carrier-state as the ability of an infected and recovered host to infect ticks, which are then able to transmit the parasite to susceptible animals. The presence or absence of circulating gametocytes (the tick infective stage) determines whether an animal can be considered a carrier at any one point in time. It has been shown that piroplasms that have recently developed within erythrocytes (i.e. within three days) are less infective to ticks than mature piroplasms (Norval *et al* 1992). Therefore, a persistently infected animal can alternate between being infective and non-infective to ticks depending on the levels and age ratios of its piroplasm population over a period of time. Demonstrating the existence of a carrier-state can only be undertaken when animals are removed from field challenge, since animals could become infected with new

antigenic strains at regular intervals. The 3.2Kb band amplified from most of the blood samples from Kitale and all from Kakamega with the TPR1 primers has not been previously reported or seen with any of the DNA samples tested at the CTVM. *T. mutans* has a universal occurrence in cattle and *T. velifera* is also very common in sub-Saharan Africa. DNA from a *T. mutans* tick derived stabilate did not amplify with either of the *Theileria* primers used and *T. velifera* was not tested. There is a possibility therefore that the piroplasms seen may be *T. velifera*, another *T. mutans* genotype or, as yet uncharacterised theilerial parasites. *T. mutans* piroplasms can be distinguished from those of *T. parva*, but the differences are very subtle and only a very experienced diagnostician could be expected to realise the differences in blood smears showing as few piroplasms as most of the smears made from the study did. Lymph node samples could be taken from the animals, as *T. mutans* can be distinguished from *T. parva* at this stage because of the larger schizonts. The schizont stage of *T. mutans* is very transient though, and is usually only seen in the initial stages of infection. If an animal had a non-clinical infection of *T. mutans*, it would be unlikely to show enlarged peripheral lymph nodes anyway, which would greatly reduce the chances of successfully sampling any schizonts.

None of the ticks in which *Theileria* was detected showed the 3.2Kb band that raises questions about the parasite transmission. If a RNA based PCR was used, the parasite lifecycle stage transcription factors between tick and bovine hosts could be so different that detection in one host and not the other may have been possible. With the DNA based approach however, it is very unlikely that the genome would change as rapidly as that; this eliminates the possibility that the bovine hosts' parasites belonged to the same population as those detected in the ticks.

The presence of the 3.2Kb band cannot be directly attributed to the presence of the piroplasms as there may have been other organisms present. High levels of eosinophils were seen in blood samples from the majority of the animals from Kitale and Kakamega, suggesting helminth infections may have been widespread. However, the specific nature of the TPR1 primers to *T. parva* strains, the detection of *Theileria*

sp. with primers 989/990 and the presence of piroplasms in all of the blood smears would tend to suggest a theilerial involvement.

PCR and bloodsmear detection of infection showed a non-significant correlation. Bath (1996) (MSc Thesis, CTVM) investigated the sensitivity of the TPR1 PCR and found it capable of detecting one *T. parva* piroplasm per 10^7 erythrocytes. He concluded that PCR analysis represented in excess of a 100 fold increase in sensitivity of detection over a bloodsmear examination in which 200 fields of view were searched. The experimental results he reported were carried out using the *T. parva* Muguga and Marikebuni strains. The primers were designed from sequence information derived from these two strains and have proved to be very reliable in amplifying them. Genotypic differences in the field parasites and/or concurrent infections in the field animals may have influenced the primer sensitivity over the levels experienced in experimental infections. A combination of the two techniques appeared to offer the maximum chance of detecting piroplasms.

PCR results from tick samples have to be carefully interpreted before they can be fully understood and put into context. To do this, it is essential to have information on the surrounding circumstances i.e. physical state of the tick, host blood infection etc. The most relevant stages to detect for the purposes of this study were the intra-salivary gland stages (sporoblast or sporozoite) which form a transmission risk, as opposed to *Theileria* present within the ingested bloodmeal, which may or may not go on to form a transmission stage at the next instar.

When DNA to be used for PCR analysis is extracted from salivary glands dissected from ticks, the results are straightforward; any amplified products obtained with specific primers represent parasites within the glands. The complication arises when DNA was extracted from dead ticks. The majority of ticks were removed from the host animals in an unengorged state. However, it is probable that some ticks would

have imbibed a certain amount of infected blood even if they do not show a noticeable expansion in size. Because the ticks were dead, dissection was not possible meaning that DNA extraction had to be carried out from whole ticks. There may have been enough parasites contained within the bloodmeal to produce a discernible band on the gel post-amplification. Knowing the infection status within the host and the condition of the tick i.e. completely unengorged, partially engorged etc. allows the risk of false positives to be reduced. There are only two compartments of the tick in which parasites may be found when the tick has attached to an animal; the gut and salivary glands. Excluding the gut as the source of parasites (based on the PCR result directly from the host blood) allowed the salivary glands to be inferred as the source of infection.

Table 4.6 shows a summary of the ticks that were PCR positive for *Theileria*. F2A1 RAm and F2A1 RAf (*R. appendiculatus* male and female) were bisected and displayed a 400bp band with TPR1 primers. The blood of the steer they were attached to showed a 3.2Kb band, suggesting that the amplified products were unlikely to have come from imbibed blood in the tick gut; therefore it is likely that salivary gland infections were being detected.

A mixed infection with *T. parva* (detected with the TPR1 primers reaction) and *Theileria* sp. (detected by the 989/990 primers reaction) was seen in two steers in Kitale. A *R. appendiculatus* sampled from a nearby farm had a mixed infection in its salivary glands. *R. appendiculatus* is the main field vector of *T. parva* and *T. taurotragi* (Norval *et al* 1992). It was shown that the TPR1 primers amplified *T. taurotragi* DNA and produced 2 bands of around 320 and 350bp (see Chapter 3). As these band sizes were not seen with TPR1 primer amplification, the *Theileria* sp. detected with primers 989/990 is unlikely to be *T. taurotragi* or the bands would have been seen when the sample was also amplifies with the TPR1 primers. Without sequencing, the possibility of identifying the parasites is somewhat limited. It is possible that the parasite represents a different strain of *T. parva* or *T. taurotragi* from the parasites in the stabilates tested with the TPR1 primers (see Chapter 3). *T.*

taurotragi is primarily a parasite of eland (*Taurotragus oryx*), although has also been shown to infect cattle, sheep and goats (Stagg *et al* 1983). Eland were not present in or near any of the areas sampled, which suggests it was unlikely the *Theileria* sp. was *T. taurotragi*. If the parasite was an uncharacterised *T. parva* strain, it may have varied to such an extent in the TPR1 region of the genome that the primers used were unable to hybridise. The TPR1 genes code for surface antigens, whereas the *Theileria* sp. primers recognise small subunit ribosomal RNA genes. Since the surface antigens of a parasite are more vulnerable to immune mechanisms than ribosomal proteins, the DNA sequences of the TPR1 region are likely to be under more selective pressure to change which would in turn affect the primer annealing characteristics.

The diagnosis of infectious disease must include information from a number of parameters. The background of the animal has to be considered in terms of prior location, contact with other animals and disease history. Clinical signs have to be monitored, and in ECf these include enlarged lymph nodes (particularly around the head, where *R. appendiculatus* feed), elevated temperature and leucopaenia. Terminal cases classically show pulmonary oedema that is displayed as acute difficulty in breathing and frothing at the nostrils. Anaemia is not usually a symptom of cattle-derived *T. parva* infection, even in terminal cases but can be a significant clinical feature in buffalo-derived *T. parva* infection. The main clinical sign of *T. mutans* infection is anaemia, the degree of which is directly related to the piroplasm parasitaemia (Young *et al* 1978). Measuring the packed cell volume (PCV) can therefore be of diagnostic value with this infection, but not with *T. parva*. The causative agent then has to be isolated. Norval *et al* (1992) defined an isolation as ''Viable organisms, isolated in experimental hosts or culture systems or prepared as a stabilate on a single occasion from a field sample.'' Measuring the level of clinical disease in the three field site areas was not the aim of this work. The primary concern was simply to identify the number of cattle and ticks carrying *Theileria* infection.

An unknown *Theileria* was detected in the salivary glands of an *A. variegatum* tick and a *R. appendiculatus* tick. Since the *Theileria* is unidentified it is not known

whether the salivary gland infections are from different species or the same. It is highly likely they will be separate species as no *Theileria* is known to be transmitted by both *A. variegatum* and *R. appendiculatus*. If they were the same species, interesting epidemiological implications would be raised.

Cattle on the KARI farms in Kitale and Kakamega were vaccinated with *T. parva* Marikebuni in 1995 and 1996. The GUTS stabilate used here was *T. parva* Marikebuni 316, the daughter stabilate of 3014 (used in Limuru). Acaricide treatment was stopped on the farm after vaccination to allow the cattle to be exposed to natural challenge from infected ticks. The animals were dipped only if the numbers of *R. appendiculatus* exceeded fifty on a half body count. The cattle were assessed one year after this regime was introduced. The numbers of clinical cases of *Theileria* were the same in vaccinated animals as in the unvaccinated controls (H. Kiara; ILRI, Kenya, personal communication). The reasons for this are not known although it is thought that a different *Theileria* species may be present in the area, against which the vaccine does not protect. No isolates of the parasites in the area have been made. ECf cases are commonly reported in farms nearby the KARI centres and low grade animals are stocked by local farmers because of the ECf risk. During the period of the field work described here, staff at the NVRC dissected seventeen *R. appendiculatus* ticks collected from the same farms at the same time as our study and found one tick to be infected with seven sporoblasts, although the parasite species identity was not ascertained.

The results from the vaccine trial in Kitale were inconclusive because of the low challenge level. The *R. appendiculatus* numbers were so low, without any form of tick control, that the animals were seen as being exposed to very little *T. parva* challenge. Only three cases of ECf out of 200 vaccinated animals were reported, compared to five cases out of 200 unvaccinated controls. During the period of the field work described here, staff at the NVRC dissected forty six *R. appendiculatus* ticks collected from the same farms and at the same time as our study and found no infection in any of them. Regular assessment of ticks collected from the KARI farm

and surrounding areas in Kitale made since that time have all proved negative for infection. This contrasts with the results presented in this chapter as 19% of the 26 *R. appendiculatus* tested were detected as being infected with *Theileria* species and 8% of these were found to be *T. parva* infections.

IFAT results on the vaccinated animals from Kitale and Kakamega a year after vaccination showed the animals had strongly seroconverted to *T. parva*, which suggests high challenge in these areas. Evidence of high challenge to cattle conflicts somewhat with the low to non-existent tick infection levels found by the NVRC in these sites. Clearly, more work is required in this area to clarify some of these issues.

Low piroplasm *T. parva* infections have been shown to produce high tick infections under laboratory conditions (Ochanda *et al* 1996). Although the high sporoblast numbers obtained under these conditions would be unlikely to occur in the field, an increase in tick infection prevalences would be expected if most or all the cattle in an area had been vaccinated with a carrier-state-inducing vaccine. The tick infection level of 1.6% in Limuru is very similar to figures reported previously from non-vaccinated areas (Walker *et al* 1981, Young *et al* 1986). This result indicates that *T. parva* vaccination may not be as effective in inducing the carrier-state as previously thought; endemic stability in vaccinated areas may be more difficult to attain. If recovered, non-carrier, animals are less well protected against challenge than carrier animals, then sustaining small holder dairy farmers' interest in a commercially available vaccine that was perceived as having limited benefit may be somewhat difficult.

The infection prevalences in ticks collected by flagging vegetation on the Muguga paddock are highly variable (H. Kiara; ILRI, Kenya, personal communication). One collection revealed 10 infected ticks out of 21 collected, whereas a collection several months later revealed only one tick to be infected out of 45 collected. The paddock is used as common grazing land for neighbouring farmers, so it may be that the infected ticks found there are a reflection of the infection levels in cattle that had grazed

there about 2 months previously (the average tick moulting time in that area). Without a more controlled system in terms of cattle movement, acaricide treatment etc. it may be difficult to gauge the impact of vaccination on the epidemiology of disease in that area.

CHAPTER 5

Quantitation of *Theileria parva* in *Rhipicephalus appendiculatus*

The aim of this chapter was to investigate quantitative aspects of the development of *T. parva* within *R. appendiculatus* in an attempt to establish the points at which they suffer the highest mortality. Even in a very highly infected tick i.e. one containing in excess of two hundred sporoblasts, there will have been a very large reduction in the number of individual *T. parva* parasites that could potentially have infected the salivary glands of that tick.

Introduction

Different incubation temperatures have been shown to affect the outcome of infection within *R. appendiculatus* on a number of occasions. It is important to assess the particular tick and parasite strains used in different laboratories because of the variation that is seen throughout. It has been reported that the *R. appendiculatus* Muguga tick isolate reared in Kenya have shorter moulting times than the same strains reared in Edinburgh and Berlin (Young and Leitch 1981a). The aim of the first experiment was to find the best environmental temperature for *T. parva* production in the tick. If one of the temperatures produced a significantly increased number of parasites, it would be useful for future experiments.

Infection levels in the tick have been studied on many occasions in relation to the immune status of cattle they fed on as a previous instar, where the immunity was directed toward the parasite or on other occasions against the tick itself. However, the immune status of the tick has been largely ignored, which may be seen as surprising, considering the longevity of ticks. Dipteran vectors are relatively short lived and so would be unlikely to show significant adaptations for acquired immunity. Arthropod immunity is regarded as having a large degree of similarity with mammalian innate, non-adaptive immunity (Hoffmann 1997). Although some elements of specificity do occur in arthropod immune responses (Nimmo et al 1997), there are no examples in

the literature of acquired immunity by arthropods *i.e.* an enhanced immune response resulting from prior sensitisation to a pathogen, such that a specific and more effective immune response can be mounted on subsequent exposure. Because ticks are so long lived in comparison with dipteran vectors, it would be of considerable selective advantage for a later instar to benefit immunologically from any infection a previous instar had encountered.

The second experiment involved a comparison of salivary gland infections in two groups of adult ticks. The first group was exposed to *T. parva* infection twice; as larvae and nymphs, whereas the other group had experienced infection only once; as nymphs. If the adult ticks in the twice infected group showed significantly lower prevalence and/or abundance values than the once infected group, the result may be explained by an acquired immune response as a consequence of encountering *T. parva* as larvae.

Ticks concentrate their bloodmeal by excreting watery saliva back into the host (Tatchell 1967). This means there are considerably more blood cells present in an engorged tick's gut than an equivalent volume of circulating blood. If the number of ingested piroplasms are to be quantified, the concentration factor of the bloodmeal must be established. Purnell (1974) reported than an engorged *R. appendiculatus* nymph will ingest 10^7 to 10^8 piroplasms but did not state the details of this calculation. These figures are accurate under certain circumstances, but variation in piroplasm levels and tick engorgement volumes necessitate a means to measure these values in individual ticks. It was important to establish the engorgement volumes of the nymphs used in this study and this was the aim of the third experiment.

A routine technique for assessing the developmental forms of *Theileria* within ticks is to produce Giemsa stained smears. This is a simple and rapid method for timing the appearance of stages in relation to the moulting times. However, accurate quantitation is not possible because of the heterogeneous distribution of the tick contents over the slide. Much of the smear is too thick to allow light through, which

means only the periphery of the smeared area can be examined. Relative comparison between different ticks or treatment groups is of course possible and the technique has proved very valuable in this regard (Young and Leitch 1981a). Electron microscopy work carried out on *Babesia* (Riek 1964, Potgieter 1976 and 1977) and *Theileria* (Mehlhorn 1978) has been invaluable for studies on parasite biology and elucidating tick-parasite interactions, but is limited in its ability to provide quantitative data over an area as large as a whole tick section. The technique used in this study involved examination by light microscopy of Giemsa stained, semi-thin (2μm) sections of whole ticks. The parasites can be quantified and viewed in the correct anatomical relationship with their host by this method.

It was anticipated that identifying parasites by light microscopy at the early stages of tick infection would be difficult because the gametes are so small and the gut so densely packed with digestive products. Therefore, the TPR1 based PCR was used (see Chapter 3) as a complementary technique which could provide information where microscopy could not. PCR analysis is particularly suitable for detecting parasites in tick faeces where very few alternative techniques could be used. If any parasites were present within the excreta, it is highly probable they would be dead and therefore difficult to identify by microscopy. Double stranded nucleic acid could still theoretically be present and relatively intact. The benefit of using the TPR1 sequence is that even if a proportion of the DNA has been digested, the high copy number will increase the probability of it being detectable by PCR analysis.

The final experiment involved the preparation of tick haemolymph smears at a time in which kinetes would be expected in that tick compartment. It is a simple technique that serves as a complementary check with the histology sections for parasite forms. This method is routinely used to assess *Babesia* infections in *Boophilus* ticks (Guglielmone *et al* 1997).

5.1. *Impact of different incubation temperatures on* T. parva *infection*

Calf 691A was infected above the RPG with 1.25t.e (150 sporoblasts) of *T. parva* Muguga Stabilate 71. The calf was treated intramuscularly with oxytetracycline on days 5 and 6 after infection at a dose of 10mg/kg body weight. Around 2000 nymphs were applied to each ear on day 9 and detached after engorgement over a three day period, the parasitaemia being <0.1 - 0.4% on the first day, 0.4 - 0.5% on the second and 0.5 - 0.3% on the third. The ticks were incubated at 18, 23 and 28^{0}C, 85% r.h for different periods of time because of the varying moulting times. At the end of the moulting period, their salivary glands were dissected out and stained with methyl green and pyronin (MGP). A group size of 20 male and 20 female ticks were chosen for each day of detachment and at each incubation temperature. The calf infection profile can be seen in Appendix 3.

5.2. *Possible acquired immunity in* R. appendiculatus

The tick batches will be referred to simply as Batch A and Batch B throughout all the lifecycle stages of the tick. Batch A ticks fed on an infected calf twice (as larvae and nymphs) and Batch B ticks only once on an infected calf (as nymphs). Both batches of ticks originated from the same randomly mixed egg batches and therefore were the same age.

5.2.a. *Feeding the larvae on different hosts - to produce Batch A and Batch B ticks*

Calf 48A was infected above the RPG with 1.25t.e (150 sporoblasts) of *T. parva* Muguga Stabilate 71. 4000 Batch A larvae were applied on day 9 (post inoculation) and detached over a piroplasm parasitaemia ranging from 3 to 22%. The calf was treated intramuscularly with oxytetracycline on days 9 - 14 after infection at a dose of 10mg/kg body weight. 4000 Batch B larvae were fed on two rabbits at the same time. Both batches then moulted to nymphs at 28^{0}C, 85% r.h. The calf infection profile can be seen in Appendix 3.

Batch A nymphs (x 265) were assessed for infection after partially feeding on a NZW rabbit. Their glands were dissected out, MGP stained and examined microscopically for the presence of parasites.

5.2.b. *Feeding the two batches of* R. appendiculatus *nymphs on a* T. parva *infected calf to compare the adult tick infections*

Calves 404 and 14500 were both infected above the RPG with 1.25t.e (150 sporoblasts) of *T. parva* Muguga Stabilate 71. Batch A nymphs (x 2000); which had previously fed on a *T. parva* infected calf and were randomly mixed from all of the larval detachment dates, were applied to the left ear of both calves and Batch B nymphs (x 2000) (that had been previously fed on a rabbit and were therefore uninfected) were applied to the right ears. The nymphs applied to calf 404 on day 6 detached during a piroplasm parasitaemia ranging from <0.1 to 8%. The nymphs applied to calf 14500 on day 7 detached during a piroplasm parasitaemia ranging from <0.1 to 5.5%. Calf 404 was treated intramuscularly with oxytetracycline on days 6 - 15 and calf 14500 on days 8 - 15 after infection at a dose of 10mg/kg body weight. The nymphs were weighed and counted as normal before they moulted to adults. The calves' infection profiles can be seen in Appendix 3.

5.2.c. *Weighing individual ticks to compare the engorgement weights of Batch A and Batch B nymphs*

Because of the difference in engorgement mass observed between the two batches during the experiment, 50 ticks from each batch were individually weighed and recorded on one particular day of detachment.

5.2.d. *Infection assessment of Batch A and Batch B adult ticks*

The adult ticks from both batches were placed in an incubator at 37^0C, 100% r.h for 5 days to allow any sporoblast infections to mature before their salivary glands were dissected out and MGP stained. The group size chosen was 20 males and 20 females unless there was an inadequate number of ticks on any particular day of detachment.

5.3. Quantifying *T. parva* stages throughout the moulting period of the tick

5.3.a. *Measuring engorgement volumes of* R. appendiculatus *nymphs*

Rhipicephalus appendiculatus nymphs (x 200) were placed in an ear bag on a NZW female rabbit and allowed to feed until they engorged and detached. After collection, the ticks were individually weighed and placed in one of 10 weight categories allocated.

Haemoglobin or other haemoproteins were estimated spectrophotometrically after being converted to pyridine haemochromogen by the technique described by Snow (1970) and Sauer and Hair (1972). 6mls of 0.1M sodium hydroxide (NaOH) were added to a glass mortar. 59 individual, engorged nymphs were bisected with a scalpel and separately placed in the liquid before being thoroughly homogenised with a glass pestle. The homogenate was then transferred to a universal tube. Sodium dithionite ($Na_2S_2O_4$) (0.8g) and pyridine (C_5H_5N) (final conc. 1.8M) were added and left for 10 mins after thoroughly mixing. The tubes were then centrifuged at 850 **x g** for 10 mins after which the clear supernatant was withdrawn and absorbance measured at 525nm on a spectrophotometer. The blank was read with the same dilutions of sodium dithionite and pyridine in 0.1M NaOH.

A standard curve was constructed from rabbit blood with which the reading from ticks could be compared. Volumes of rabbit blood were added to the NaOH in 5µl increments from 5 to 50µl and processed as described above. The blood did not require homogenisation as it immediately lysed in the sodium hydroxide solution. The spectrophotometer readings were plotted on a graph against the blood volume they were derived from. The blood volume the ticks had ingested was then extrapolated from this graph with the spectrophotometer readings from the ticks which meant another graph could be drawn of actual tick weight plotted against the volume of blood ingested (as gauged from the previous graph).

5.3.b. *Assessing* T. parva *numbers within semi-thin sections of* R. appendiculatus *by light microscopy*

Calf 48A (referred to previously) was used to feed nymphs for this part of the experiment. The nymphs detached over a four-day period with the parasitaemia ranging from 3.2 to 22.4%. Ticks were sampled at regular intervals of one to two days from days 0 to 22 post-detachment. During the moult they were incubated at 28^{0}C, 85% r.h. A full control series of ticks was prepared at the same time. The nymphs were from the same batch as those used for the calf feed, but were fed on a rabbit. In addition, a series of histological sections of *R. appendiculatus* nymphs fed on uninfected calves was available from a previous study in this laboratory (Walker and Fletcher 1987). Ticks were taken from whatever batches that were available (see Table 5.2) for processing. The number of ticks that detached on the first day was insufficient to include in the histology part of the work. After the ticks had moulted and completed post-moult development, an infection assessment of the adult salivary glands was carried out. Twenty males and twenty females were selected from each day of detachment unless there were insufficient numbers. A complete control series was prepared with rabbit fed nymphs (taken from the same batch as those used for the calf feed). The control feeds were carried out at the same time as the calf feed to ensure the ticks were of the same age when they fed.

The ticks were stuck to wax in a glass Petri-dish and dissected under 1% saline solution. Once the scutum had been removed, a small volume of 0.1M Karnovsky's fixative (see Appendix 6 for details), pH 7.2 was squirted over the viscera to partially fix them *in situ* and make the dissection process easier. The viscera were then removed from the exoskeleton and placed in Karnovsky's fixative at 4^{0}C. At around day 12 post-detachment, the developing adult ticks were dissected from their nymphal exoskeletons, then re-stuck in wax and dissected separately. After a month in fixative, the ticks were removed and prepared for methacrylate embedding. After washing in 0.1M phosphate buffer (see Appendix 6) for 3 x 20 mins, they were dehydrated in 3 to 4 changes of 100% acetone for one hour at 4^{0}C. Infiltration with the monomer of a water miscible plastic - hydroxyethylmethacrylate (Jung Historesin

Plus, Leica Microsystems U.K Ltd.) was carried out for 5 days at 4^0C. The samples were constantly rotated during all the steps from washing to infiltration. The embedding solution was prepared by adding 0.5ml of the proprietary hardener to 15ml of infiltration solution and mixing thoroughly at 4^0C. The mixture was dispensed into 1ml flat bottomed, cylindrical moulds at 4^0C and 3 - 5 ticks were then placed on top of the liquid and allowed to sink to the bottom of the mould. The resin was allowed to polymerise overnight at 4^0C, after which the moulds were removed and the resin dried out on a warming plate. Sections were cut at 1 to 2μm on an ultra-microtome and floated on a small volume of water before drying onto the slide. They were stained for 15 mins in 5% Giemsa's stain before being mounted in plastic mountant (DePeX, Gurr, England).

The advantage of examining parasites in section is that their morphology is more representative of their true state than if they were smeared on a slide. Because smears are dried before being stained, cells can shrink and become distorted. Published images and measurements from light and electron microscopy (Schein *et al* 1977, Mehlhorn *et al* 1978) were used as guides throughout examination of the sections. Sexual forms were easily recognisable, as their shape was very distinctive and dissimilar to tick tissue. Mature zygotes were more difficult to distinguish from protein vesicles in the gut digestive cells because they were of a similar size. However, they were highly vacuolated and contrasted with tick tissue on the basis of colour as they stained differently. As zygotes were roughly spherical, they presented a more uniform section than kinetes, which were long and tapered in comparison. Even if there was an element of doubt regarding whether a zygote or kinete was being presented in section, the morphological structure seen within the objects clearly distinguished them from tick digestive products. The sections of infected ticks from each day of sampling were constantly compared with those of control, uninfected ticks to ensure accurate identification.

A number of tick samples fixed in Karnovsky's were prepared for viewing by transmission electron microscopy. It was hoped the higher magnification images

could provide information that would be unavailable at the level of light microscopy. A detailed protocol for electron microscopy can be seen in Chapter 6.

5.3.c. *PCR detection of* T. parva *in the tick gut, faeces and salivary glands*

Calf 58B was infected above the RPG with 2t.e (156 sporoblasts) of *T. parva* Marikebuni Stabilate 72. The *T. parva* Marikebuni isolate was used in this experiment because detached ticks were available from another experiment being conducted at the same time which assessed the infectivity of the isolate to ticks (D. Brown; CTVM, personal communication). Results from Chapter 3 demonstrated that PCR detection was equally sensitive to all strains of *T. parva* tested, including *T. p* Marikebuni. For this reason, *T. p* Marikebuni was regarded as entirely appropriate for use in a PCR detection study. Nymphs (x 500) were applied to the ear of the infected calf on days 8 and 9 post infection, and detached during a piroplasm parasitaemia ranging from 0.5 to 2.8%, after which they were incubated at 28^0C, 85% r.h. Dissections were carried out under 1% saline and the guts and salivary glands were removed. Procedures to prevent cross-over contamination from instruments were as described in Chapter 3. Ten ticks were dissected on days 0, 5, 7, 12, 14, 22, 26 post-detachment and five ticks on days 16 and 20 post-detachment. The calf infection profile can be seen in Appendix 3.

When a number of ticks were removed from the universal tube on day 5 post-detachment, a sample of faeces was removed at the same time. 0.01, 0.001 and 0.0001g of faeces were weighed out and dissolved in 200µl of PBS. This was then added separately to the lysis buffer from the DNA extraction kit. The haematin granule weighing 0.0001g had been excreted by a single tick. The samples were processed for DNA extraction and PCR analysis as described in Chapter 3.

5.3.d.1. *Examining haemolymph smears to check for the kinetes*

The nymphs that had engorged on calf 404 were used. Haemolymph smears were made from 100 ticks to check for the presence of kinetes. Fifty day 15 and fifty day 16 (post detachment) ticks were used as the histology sections indicated this to be the

most likely time to see kinetes. Ticks were held with forceps between their dorsal and ventral surfaces. The tarsus (distal segment) of one of their back legs was removed with a scalpel and the haemolymph from the wound was smeared across the slide by moving the body of the tick. Care was taken to avoid contamination with gut contents, and if smears revealed contamination, they were rejected.

5.3.d.2. *Haemocyte counts*

Haemocyte counts were carried out at the same time. The granular haemocytes were usually broken up by the smearing process, leaving intact, purple staining nuclei that were recorded.

5.3.e. *Examining the structure of developing salivary glands*

Salivary glands were dissected from the ticks during the moulting process to give an indication of any changes the organs were going through and to indicate the morphology of the glands when kinete penetration would be occurring.

All statistics were carried out using the INSTAT© computer program (Graphpad Software, Inc.). The data from these experiments were not normally distributed; thus they were analysed using non-parametric, two-tailed Mann-Whitney U-tests (unless otherwise indicated) which compared the differences between group medians.

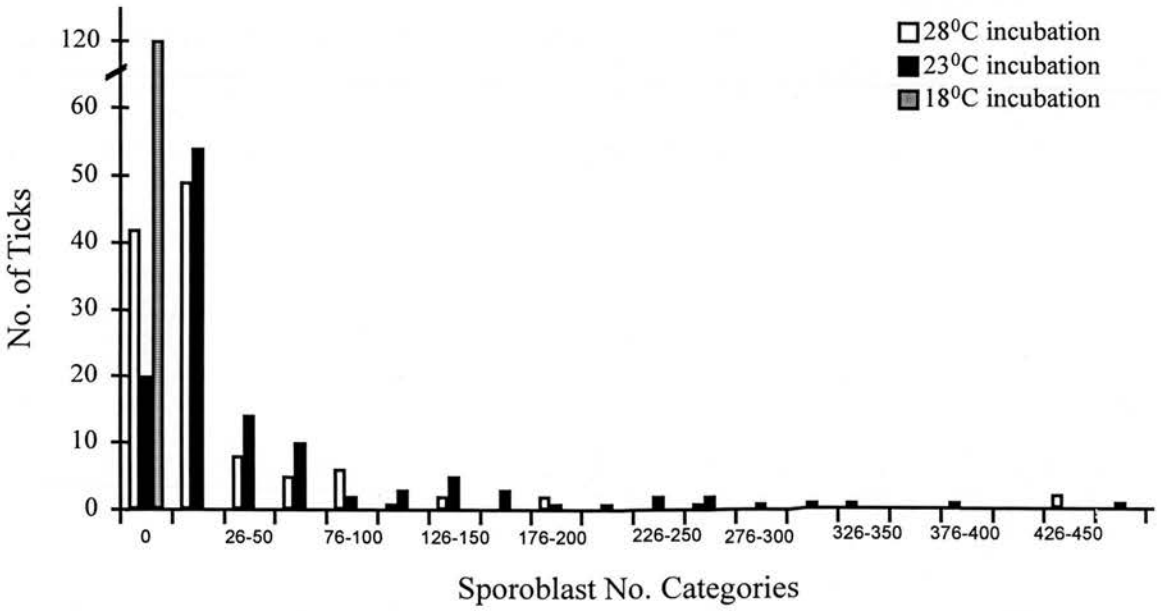
Results

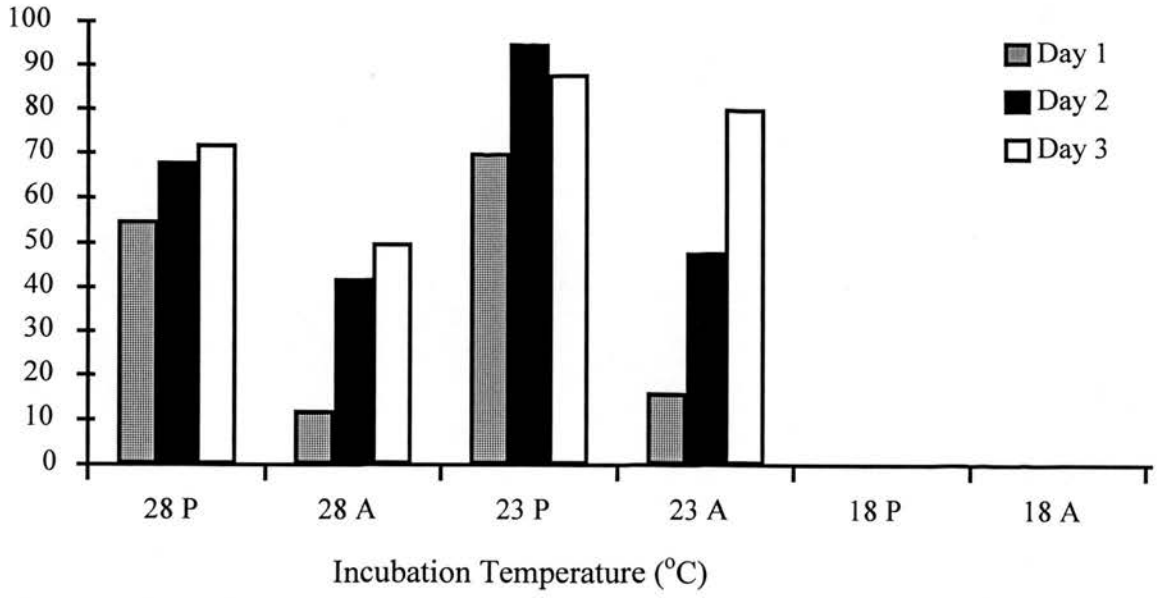
5.4. *Impact of different incubation temperatures on* T. parva *infection*

A mild infection was produced in calf 691A with the piroplasm parasitaemia reaching a maximum of 0.5%. The different incubation temperatures of the ticks fed on this calf resulted in a marked contrast in tick and parasite development. At 28^0C, the ticks took 14 days from detachment to eclosion from the nymphal exoskeleton and another 14 days to complete post-moult development. At 23^0C, the eclosion and post moult times increased to 28 days and at 18^0C, both times increased to 56 days. The raw data for the tick infections from this experiment can be seen in Table 3A, Appendix 3.

Figure 5.1. Graph showing the frequency distribution of sporoblast numbers in adult tick salivary glands after nymphs had moulted at 3 different temperatures; 18^0C, 23^0C and 28^0C. 120 ticks were included per group. No infection was present in any of the 120 salivary glands from ticks incubated at 18^0C. The ticks incubated at 23^0C contained more than twice the number of sporoblasts compared with those incubated at 28^0C.

Figure 5.2. Graph showing the prevalence and abundance data from sporoblast numbers in tick batches incubated at 18, 23 and 28^0C. A: abundance, P: prevalence. Piroplasm levels on detachment days were: Day 1: <0.1 - 0.4%, Day 2: 0.4 - 0.5%, Day 3: 0.5 - 0.3%. 20 male and 20 female ticks were included in each group. The scale on the y-axis refers to sporoblast numbers (abundance) and % of ticks infected (prevalence). Abundance refers to the average number of *T. parva* sporoblasts per tick.


28⁰C incubation
23⁰C incubation
18⁰C incubation
No. of Ticks
120
60
50
40
30
20
10
0
0
26-50
76-100
126-150
176-200
226-250
276-300
326-350
376-400
426-450
Sporoblast No. Categories


Day 1
Day 2
Day 3
100
90
80
70
60
50
40
30
20
10
0
28 P
28 A
23 P
23 A
18 P
18 A
Incubation Temperature (°C)

incubated ticks because they have a larger number of infections in the region of 126 to 300 sporoblasts than the 28°C incubated ones. Fig. 5.2 shows the prevalence and abundance data for the three groups. Ticks incubated at 23°C showed a significantly increased level of infection over those incubated at 28°C ($P < 0.01$).

5.5. *Possible acquired immunity in* R. appendiculatus

The prevalence and abundance data from two batches of adult ticks were compared. Batch A ticks had experienced *T. parva* infection as larvae and nymphs, whereas Batch B ticks had experienced infection only as nymphs.

5.5.a. *Feeding the larvae on different hosts - to produce Batch A and Batch B ticks*

Salivary glands from 265 of Batch A nymphs were assessed for *T. parva* infection. No infection was found in any of the glands. Examining the salivary glands from Batch B ticks was unnecessary because the larvae were fed on a rabbit and therefore had not encountered *T. parva* infection.

5.5.b. *Weighing individual ticks to compare the engorgement weights of Batch A and Batch B nymphs*

The engorgement weights of the ticks were notably different between the two batches as can be seen in Table 5.1. Batch A nymphs were consistently smaller than Batch B nymphs on every day of detachment from both calves. Fifty Batch A and B nymphs were individually weighed from calf 404 on one particular detachment day. Fig. 5.3 shows the distribution of values on that day. An unpaired t-test with Welch correction showed an extremely significant difference between the means of the two batches ($P < 0.0001$). The raw data from this experiment can be seen in Table 3B, Appendix 3.

5.5.c. *Infection assessment of Batch A and Batch B adult ticks*

A large difference was noted between the sporoblast infections in the two batches. Batch B adult ticks (once infected) were significantly more infected than Batch A adult ticks (twice infected) ($P < 0.0001$). Fig. 5.4 shows a frequency distribution of

Table 5.1. Mean engorgement weights of Batch A and Batch B nymphs recorded from calves 404 and 14500 over the entire period of detachment. Batch A nymphs had been infected with *T. parva* previously as larvae, whereas Batch B nymphs had experienced *T. parva* infection for the first time as nymphs as they had fed on rabbits as larvae. / represents a day when no detachment occurred.

Figure 5.3. Graph showing the distribution of engorgement mass of Batch A and Batch B nymphs. Both batches had fed as nymphs on *T. parva* infected calf. Batch A larvae had fed previously on a *T. parva* infected calf whereas Batch B larvae had fed on rabbits. n = 50.

Day of detachment	Mean weight (mg)	
	Batch A	Batch B
1	/	7.6
2	4.9	8.6
3	5.0	8.2
4	5.0	9.0
5	5.0	8.4
6	5.1	7.8

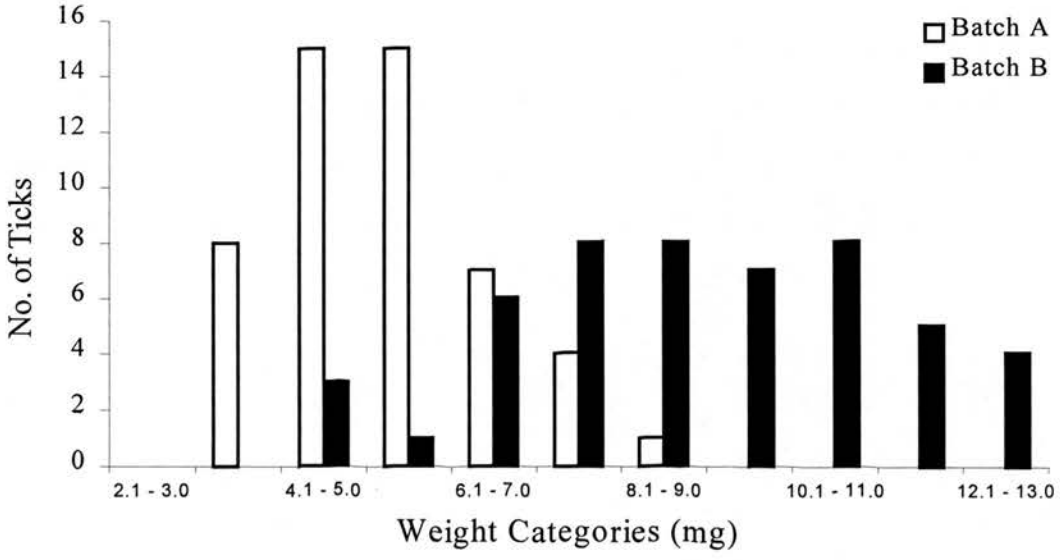
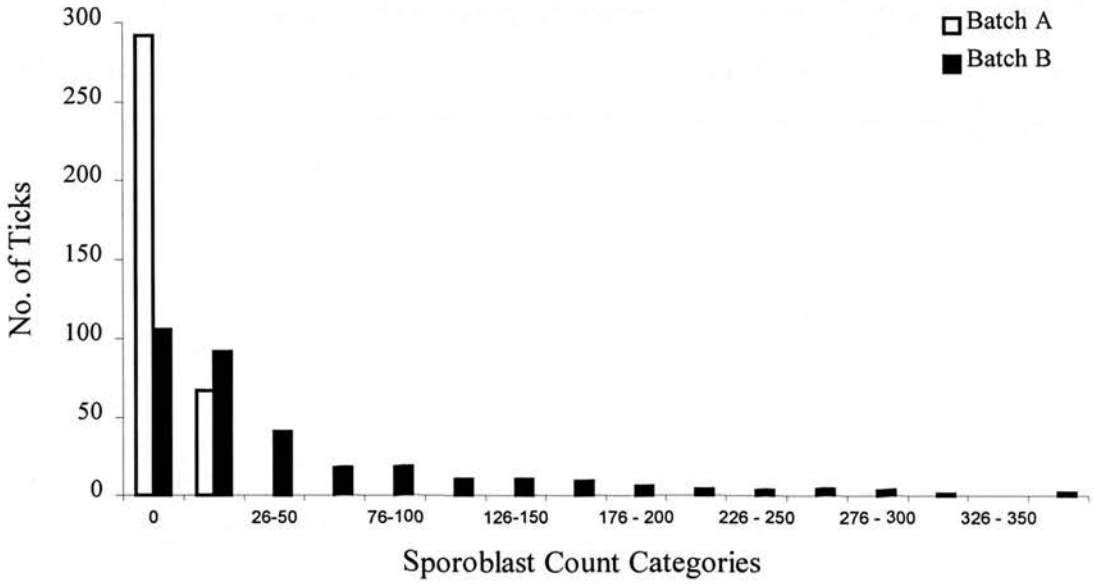


Figure 5.4. Graph showing a frequency distribution of *T. parva* infection in salivary glands of Batch A and Batch B adult ticks. Batch A ticks (exposed to *T. parva* infection as larvae and nymphs) were significantly less infected than Batch B ticks (exposed to *T. parva* infection only as nymphs).

Figure 5.5. Graph showing the prevalence and abundance data from sporoblast numbers in salivary glands from Batch A and Batch B adult ticks. A: abundance, P: prevalence. The scale on the y-axis refers to sporoblast numbers (abundance) and % of ticks infected (prevalence). 20 males and 20 females were included in each group.


Batch A
Batch B
No. of Ticks
300
250
200
150
100
50
0
0
26-50
76-100
126-150
176 - 200
226 - 250
276 - 300
326 - 350
Sporoblast Count Categories

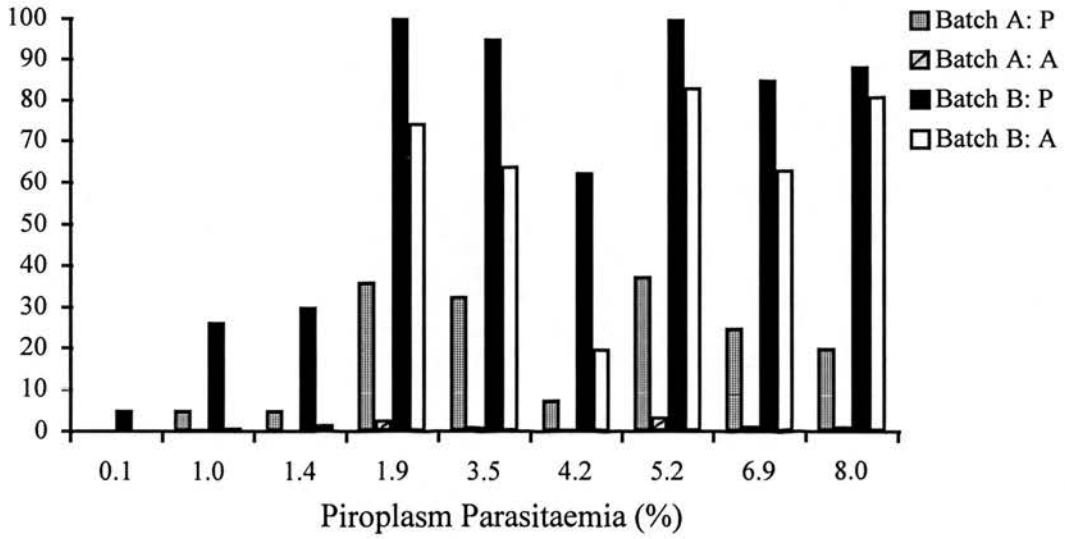
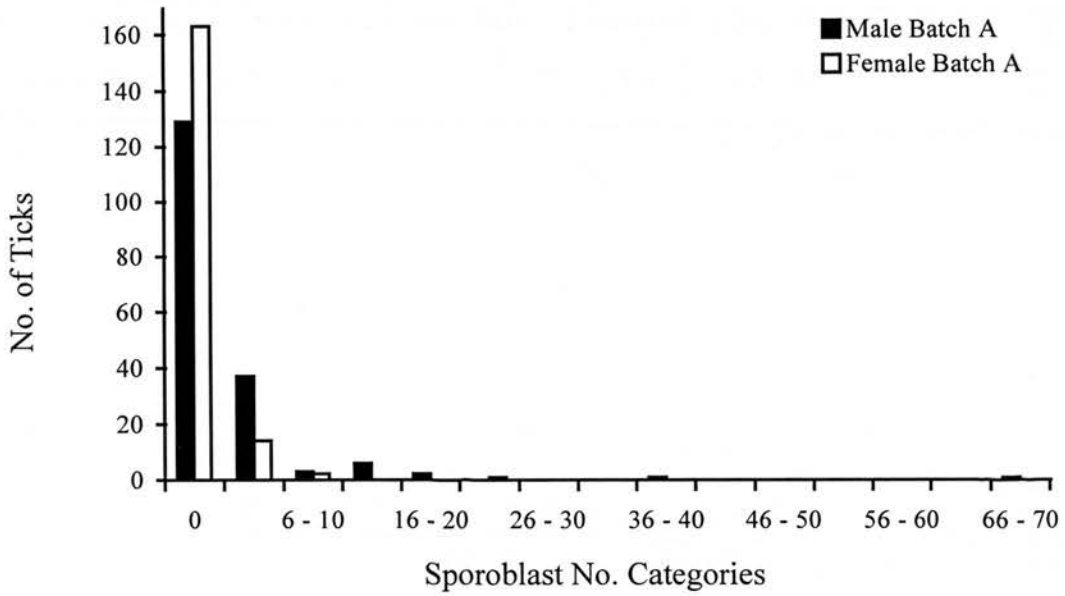
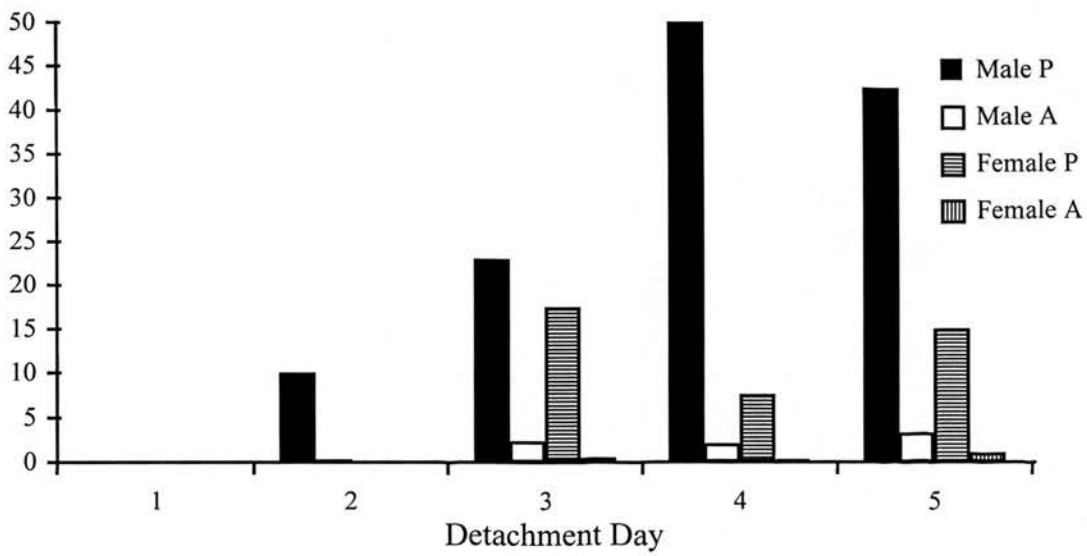

Batch A: P
Batch A: A
Batch B: P
Batch B: A
100
90
80
70
60
50
40
30
20
10
0
0.1
1.0
1.4
1.9
3.5
4.2
5.2
6.9
8.0
Piroplasm Parasitaemia (%)

Figure 5.6. Graph showing a frequency distribution of salivary gland infections in Batch A male and female ticks. Both sexes were exposed to *T. parva* infection as larvae and nymphs. Males were significantly more infected than females.

Figure 5.7. Graph showing the prevalence and abundance data from sporoblast numbers in salivary glands from Batch A male and female ticks. A: abundance, P: prevalence. The scale on the y-axis refers to sporoblast numbers (abundance) and % of ticks infected (prevalence). 20 males and 20 females were included in each group.


Male Batch A
Female Batch A
No. of Ticks
160
140
120
100
80
60
40
20
0
0
6 - 10
16 - 20
26 - 30
36 - 40
46 - 50
56 - 60
66 - 70
Sporoblast No. Categories


50
45
40
35
30
25
20
15
10
5
0
Male P
Male A
Female P
Female A
1
2
3
4
5
Detachment Day

their infection profile and Fig. 5.5 shows prevalence and abundance data for the two batches. The raw data from this experiment is displayed in Table 3C, Appendix 3.

Batch A male ticks contained significantly higher infection levels than Batch A females ($P < 0.01$). Fig. 5.6 shows a frequency distribution of their infection levels. Figs. 5.7 shows the prevalence and abundance data for both sexes. No significant difference was found between the infection profile in Batch B male and female ticks.

It was noted upon dissection and examination of the glands was a brown deposit left on some of the acini, specifically those that were in direct contact with the gut. Both tick batches from calf 404 showed a putative phenoloxidase enzyme effect, but the glands in ticks from calf 14500 actually had a gut derived deposit on top of them, as if the guts had become perforated and allowed a certain amount of their contents to escape. This material was much more granular in nature and tended to accumulate in the clefts between the acini, whereas the phenoloxidase type staining tended to be a thinner, more homogeneous covering over certain acini and even specific parts of some acini. Batch A and B ticks from calf 404 showed a difference in the numbers that demonstrated the phenoloxidase type effect. Batch A had markedly higher levels, particularly in ticks which detached as nymphs at higher piroplasm parasitaemias. Sixteen percent of Batch A and one percent of Batch B ticks were affected. A significant difference was noted between Batch A and B ($P < 0.05$), but a non-significant difference was noted in the occurrence of phenoloxidase between the male and female glands in Batch A. A small difference was seen between the gut deposited material between Batch A and Batch B ticks from calf 14500. Six percent of Batch A and nine percent of Batch B ticks were affected, but this difference was found to be non-significant.

It was noted that there was marked variation in the state of sporoblast maturity within Batch A adult ticks (exposed to *T. parva* infection as larvae and nymphs) in particular.

This variation was noted between ticks and also within individual ticks' salivary glands. Some were dark purple spheres (mature) while others, even on the same gland, were thin, semi circles of mid red colour (immature) (data not shown).

5.6. Quantifying *T. parva* stages throughout the moulting period of the tick

5.6.a. *Measuring engorgement volumes of* R. appendiculatus *nymphs*

Fig. 5.8 shows the absorbance values obtained from measured volumes of rabbit blood. Fig. 5.9 shows tick weight and the corresponding volume of blood as judged from Fig. 5.8.

The concentration of the bloodmeal can be worked out using the equation:

$$\frac{(1.05)\ \text{blood volume equivalent (µl)}}{\text{mass of tick due to engorgement (mg)}}$$

1.05 - specific gravity of blood

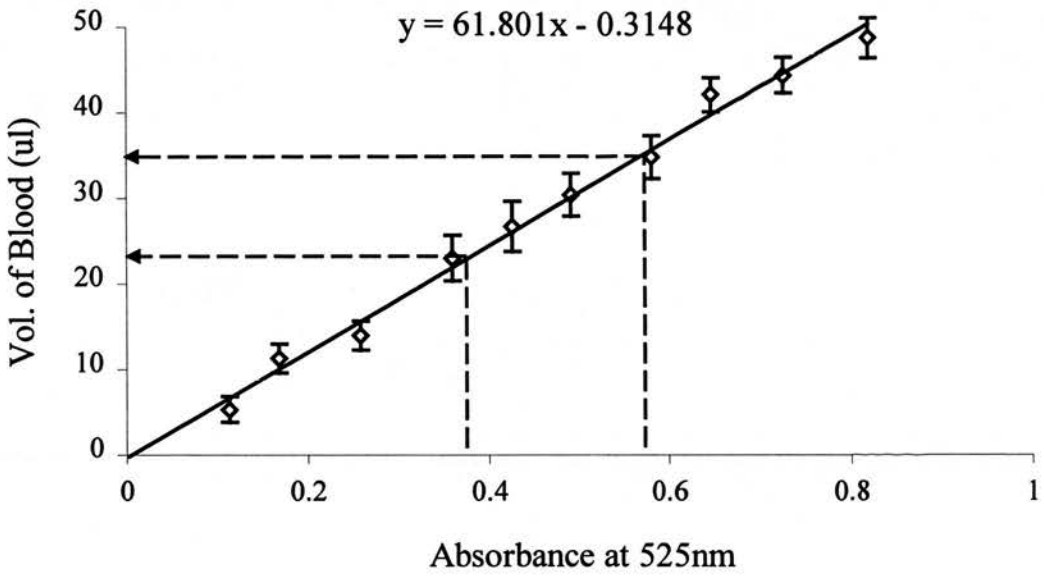
The mean was taken for 10 separate mass groups and the figures used in the equation. The mean of this figure was 2.73, i.e. the *R. appendiculatus* nymphs concentrated their bloodmeal by a factor of 2.73 times.

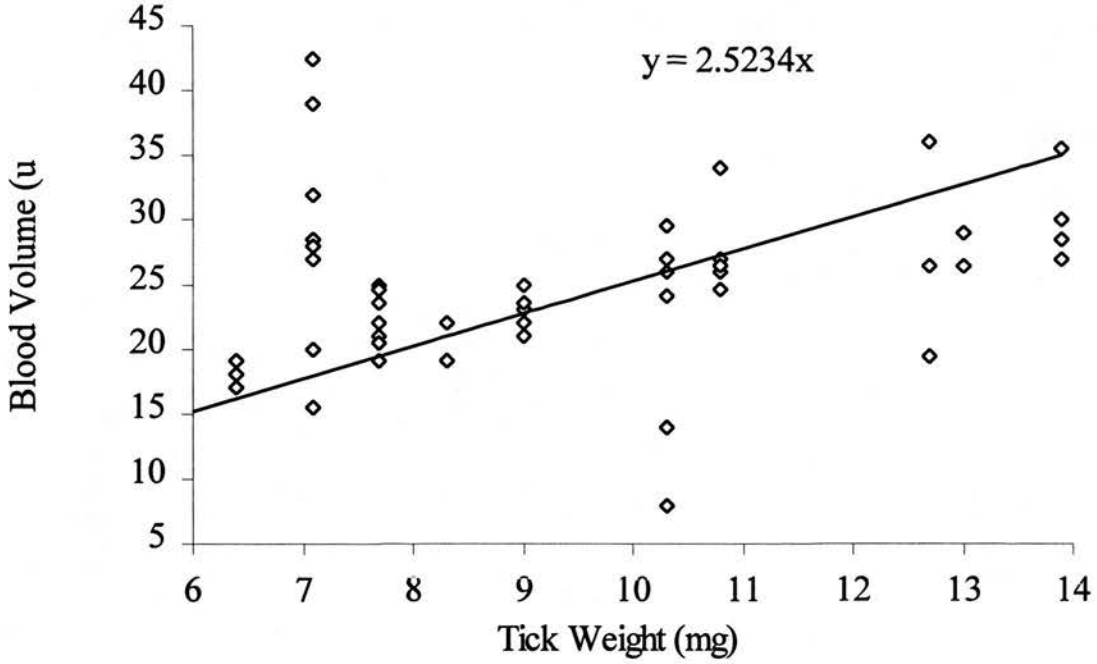
5.6.b. *Assessing* T. parva *numbers within semi-thin sections of* R. appendiculatus *by light microscopy*

Exoerythrocytic piroplasms and sexual forms were seen in the gut on day 0 post-detachment amongst the amorphous protein mass that contained a proportion of intact erythrocytes. Large clumps of piroplasms were seen (which accounted for the majority of forms) although individual sexual forms could be distinguished, suggesting a very rapid transformation from piroplasms (Figs. 5.10a - c). Delicate focusing was required as the fine microtubules were difficult to see against the darkly basophilic protein. By day 2, the protein was seen to become vesicle bound in sessile digestive cells lining the gut wall and lumenal spaces had appeared. These areas were largely protein free and contained a glutinous, nutrient milieu that would later be seen to be vesicle bound. The majority of sexual stages were seen in the gut lumens as opposed to the digestive cells. The small aggregations of sexual forms were less

Figure 5.8. Graph showing a standardisation curve for (525nm) against a range of rabbit blood volumes. The dotted lines indicate how the nymphal bloodmeal engorgement volumes were extrapolated from the standard line generated from the measured volumes of rabbit blood. Error bars show the standard deviations.

Figure 5.9. Graph showing the extrapolated bloodmeal volumes of a batch of rabbit fed nymphs. The engorged nymphs were divided into weight categories and processed as described in the text. The spectrophotometer values obtained at 525nm were then plotted against the graph in Fig. 5.8, after which the equivalent ingested blood volumes could be extrapolated.


y = 61.801x - 0.3148
Vol. of Blood (ul)
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Absorbance at 525nm


y = 2.5234x
Blood Volume (u
45
40
35
30
25
20
15
10
5
6
7
8
9
10
11
12
13
14
Tick Weight (mg)

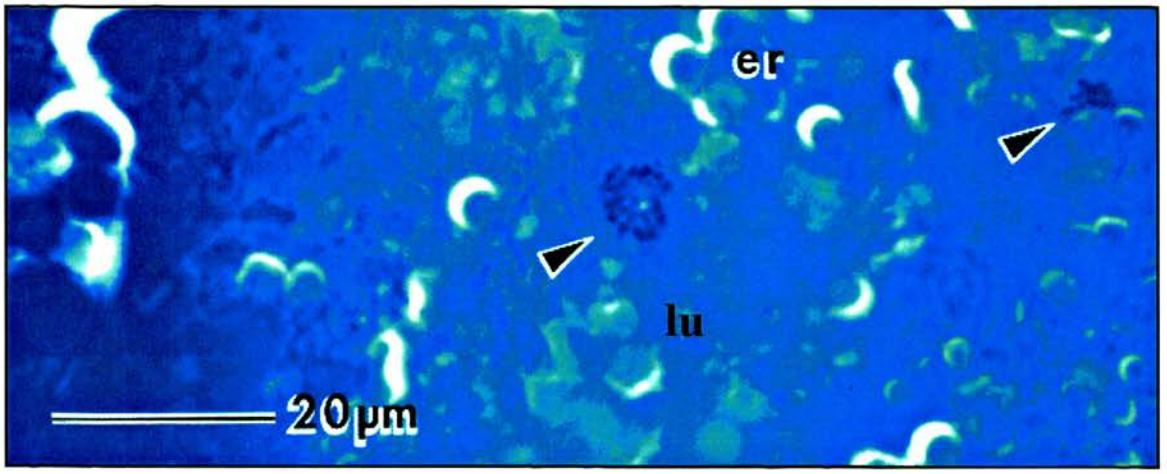
frequently encountered and individual forms became more commonly seen (Figs. 5.10d - f). No parasite forms were seen between days 2 and 8 post-detachment. Zygotes were seen on day 8 and gradual development was clearly visible (Figs 5.10g - j) through to the appearance of kinetes (Figs 5.10k - m) by day 15. As the zygotes matured and began transforming to kinetes they became more intensely stained. The kinetes were clearly recognisable in longitudinal section as they are long and thin, but less so in transverse section. Kinetes were only denoted as such if a longitudinal section was seen, therefore some of the images of kinetes in transverse section may have been classed as zygotes leading to an underestimation of their numbers and an overestimation of zygote numbers. In the histological sections, kinetes were only seen in the gut and in no other tick compartment. Sporonts were first detected on day 17 (Figs 5.10n -o) and were clearly distinguished from the acinar granules in the e-cells of Type III acini, where they were seen to develop exclusively, and later became immature sporoblasts by day 21 (Fig. 5.10p). During the dissection process, it was probable that some of the haemolymph was lost in the saline solution. However, as Fig. 5.11 shows, large numbers of haemocytes were retained around the viscera and they (and consequently, the haemolymph) were present in every section examined.

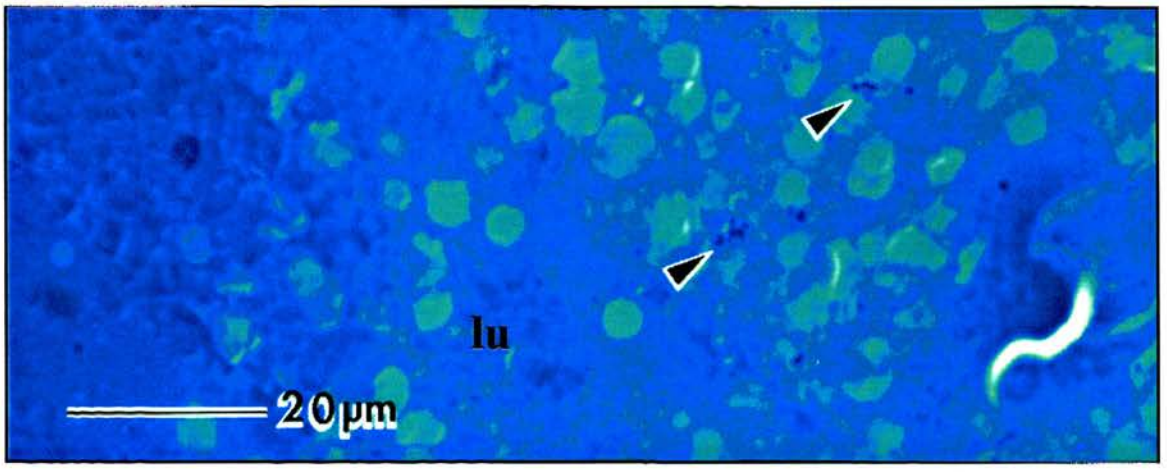
Evidence from light microscopy suggested that zygotes and kinetes may have been suffering pathological effects in the gut digestive cells (Figs. 5.12a and b). The colour differentiation from the protein vesicles in the gut was a useful means by which to identify the zygotes. The zygotes shown in 5.12a were typical of several forms seen in a ticks at the later stages of digestion (~ days 15/17). Their stained colour resembled the rest of the digestive contents and they usually appeared partially disintegrated. The image presented in Fig 5.12b is difficult to interpret, but may indicate a kinete being digested or destroyed in some way. The colour and shape were typical of a kinete, but the highly vacuolated appearance (which was not seen in the majority of kinetes), indistinct shape and the surrounding protein vesicles suggest it may have been the target of destructive processes. Electron microscopy was used to try to further elucidate the images. A limited number of parasites were detected by

Figure 5.10a. Aggregations of *T. parva* piroplasms in the gut of a day 0 post-detachment nymph. The arrows point to clumps of piroplasms in the gut lumen (lu). Many erythrocytes (er) are still intact.

Figure 5.10b. Individual piroplasms and sexual stages in the gut lumen of a day 0 post-detachment nymph. The arrows point to individual parasite forms. lu - lumen.

Figure 5.10c. Individual sexual stages in the gut lumen of a day 0 post-detachment nymph. The gametes have relatively large nuclei and long, very fine microtubules (arrows). The protein is becoming vesicle bound (pv) and the nucleus of a developing sessile digestive cell can be seen (n). lu – gut lumen.


er
lu
20µm


lu
20µm

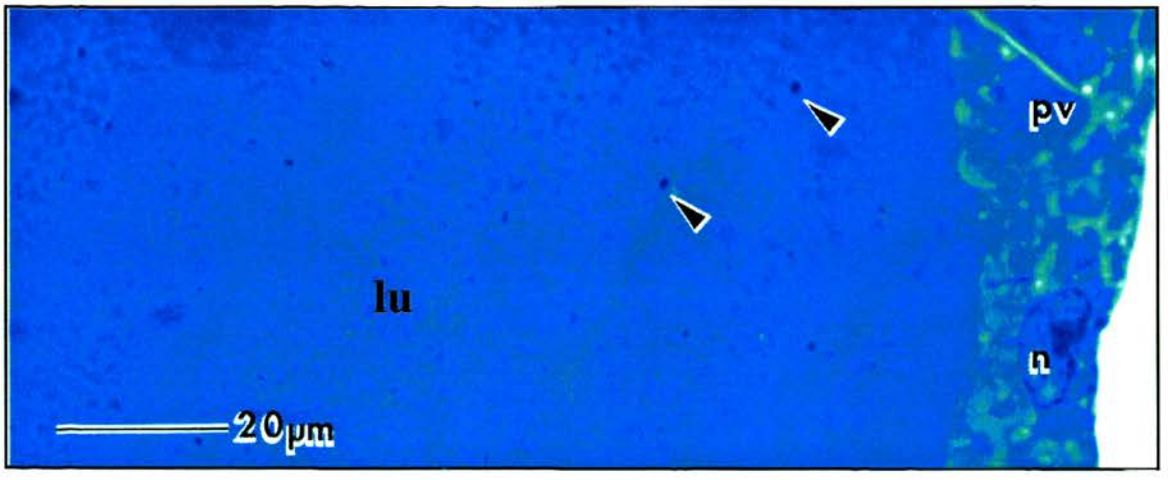
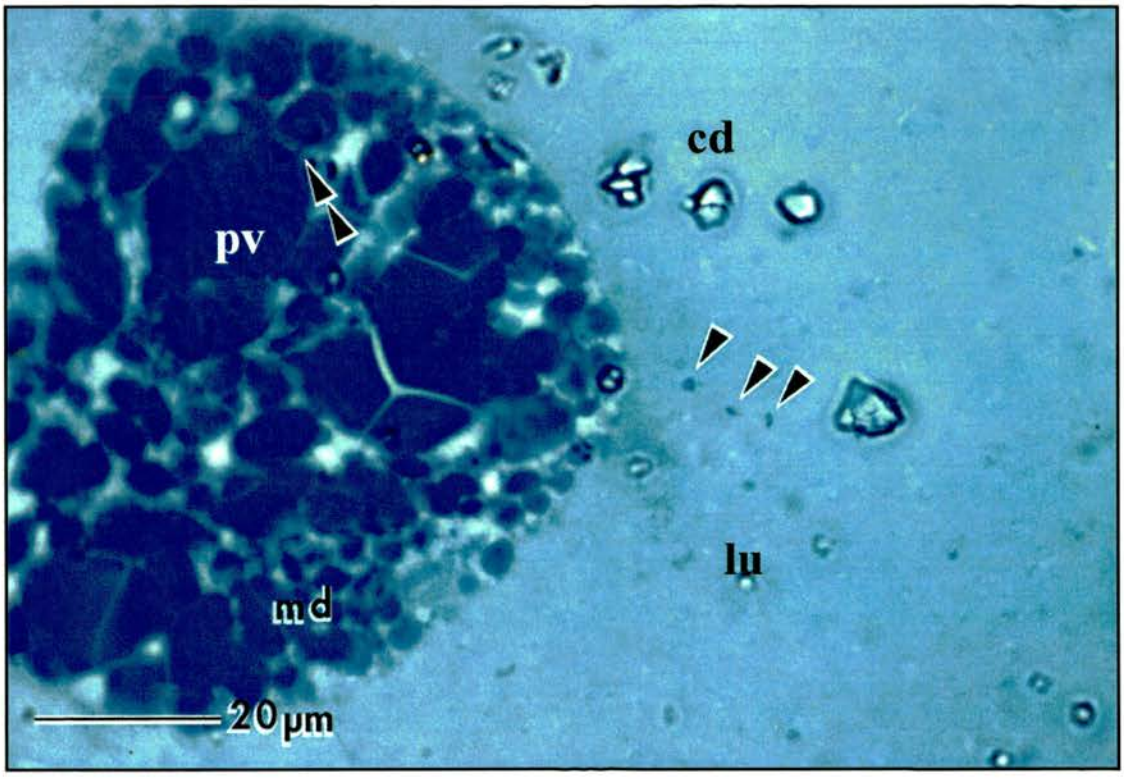
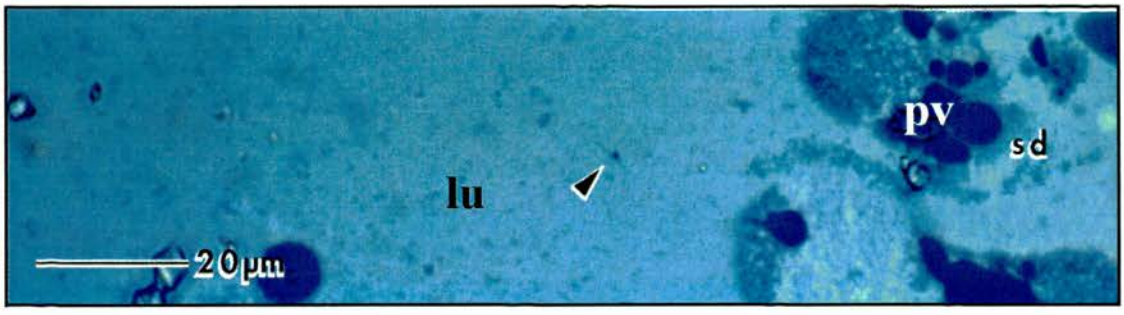

pv
lu
n
20µm

Figure 5.10d. Individual sexual stages in the gut lumen of a day 2 post-detachment nymph. Arrows point to the gametes beside a motile digestive cell (md). The gamete on the left is binucleate. The double arrows may point to piroplasms that were incorporated within protein vesicles in the digestive cell. A crystalline deposit that represents an artifact of processing can be seen over parts of the slide (cd).

Figure 5.10e. Strallenkorper in the gut lumen of a day 2 post-detachment nymph. The arrow points to the three, very fine microtubules radiating from the large, central nucleus. Protein vesicles (pv) are beginning to form within the sessile digestive cells (sd). lu – lumen.

Figure 5.10f. Aggregation of gametes in the gut lumen of a day 2 post-detachment nymph. A sessile digestive cell (sd) is immediately below the small clump of gametes (arrowed). lu – lumen.


cd
pv
lu
md
20µm


pv
sd
lu
20µm

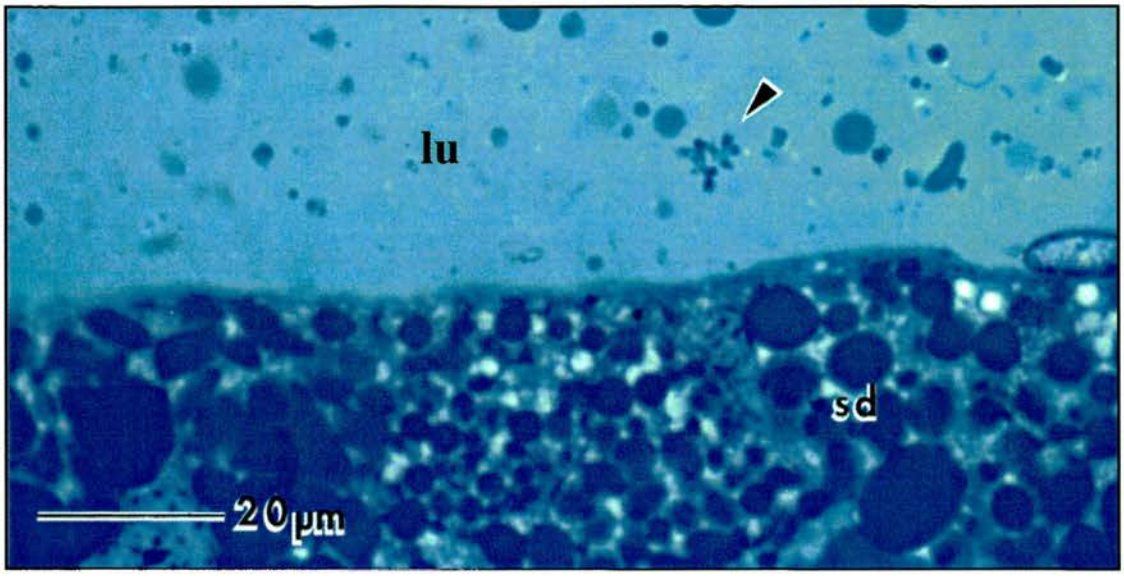
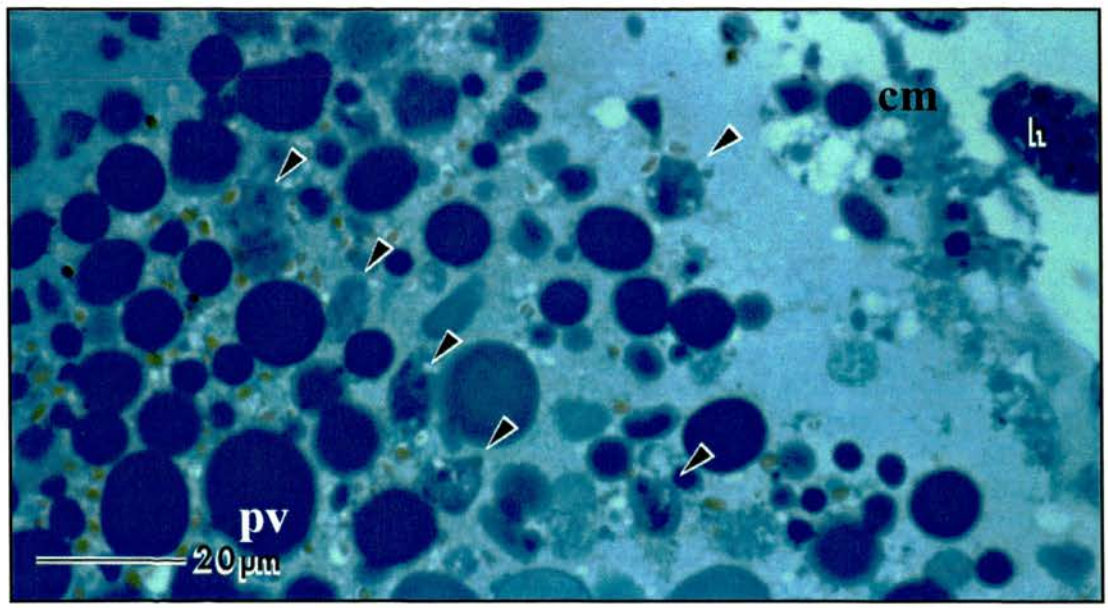
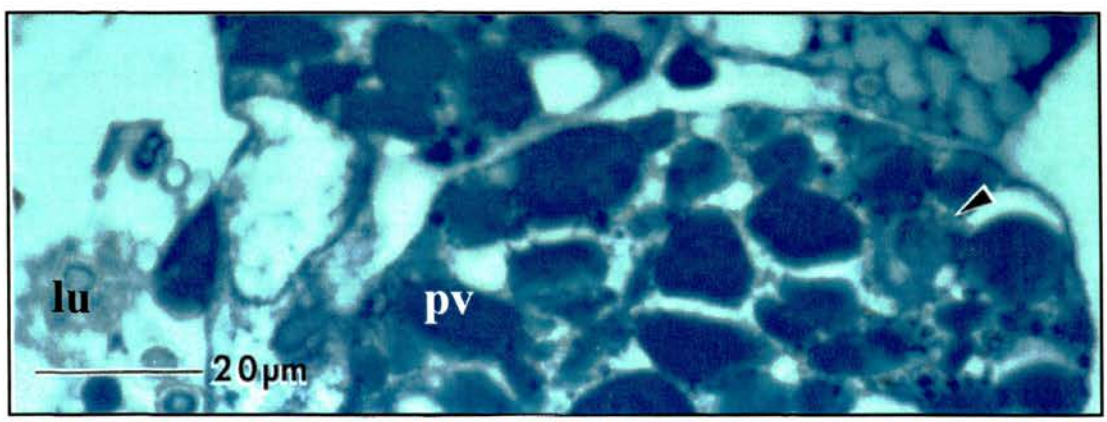

lu
sd
20µm

Figure 5.10g. Zygotes in a gut digestive cell in a day 8 post-detachment nymph. The section pictured was from a very highly infected tick. pv – protein vesicle, cm – gut digestive cell membrane, h - haemocyte present in haemolymph surrounding the gut.

Figure 5.10h. Zygote in a gut digestive cell in a day 10 post-detachment nymph. The arrow shows a developing zygote/kinete structure within the outer zygote membrane. A large vacuole can be seen surrounding the developing kinete. pv – protein vesicle, lu – lumen.

Figure 5.10i. Zygote in a gut digestive cell in a day 13 post-detachment nymph. The arrow points to the more basophilic and clearly defined structure. The nucleus can be discerned at what would have become the anterior end of a developing kinete. The parasite has been incorporated within a motile digestive cell (md) which would have earlier separated from a sessile digestive cell (sd) around the gut wall. pv – protein vesicle, lu – lumen.


cm
lu
pv
20µm


lu
pv
20µm

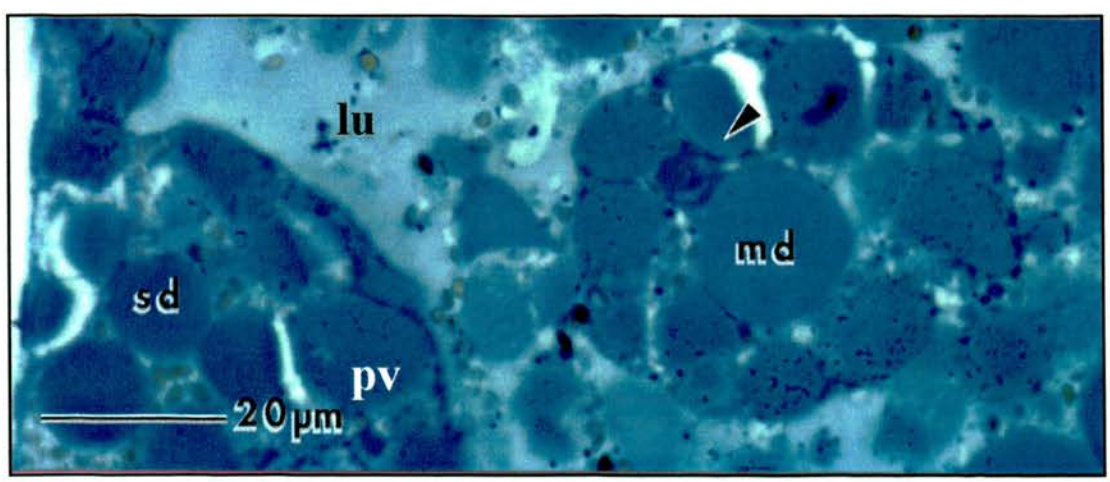
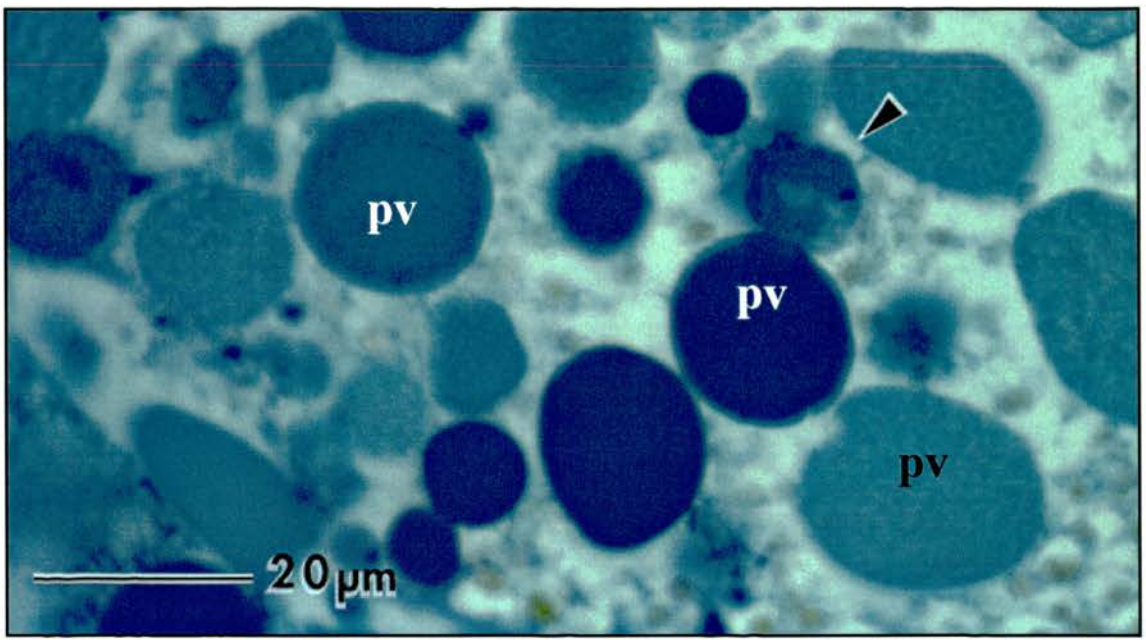
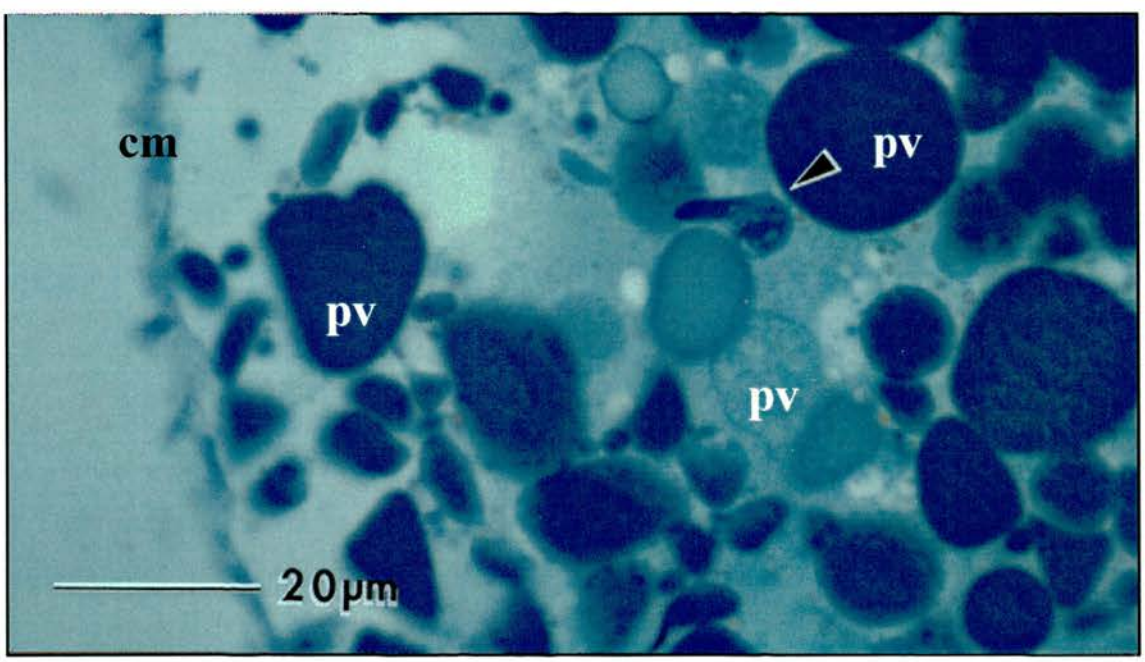

lu
md
sd
pv
20µm

Figure 5.10j. Mature zygote/kinete in a gut digestive cell in a day 14 post-detachment nymph-adult tick. pv – protein vesicle.

Figure 5.10k. Emerging kinete in a gut digestive cell in a day 14 post-detachment nymph-adult tick. cm – digestive cell outer membrane, pv – protein vesicle.

Figure 5.10l. Kinete in a gut digestive cell in a day 15 post-detachment adult. cm – digestive cell outer membrane, pv – protein vesicle, rb – residual bodies.


pv
pv
pv
20µm


cm
pv
pv
pv
20µm

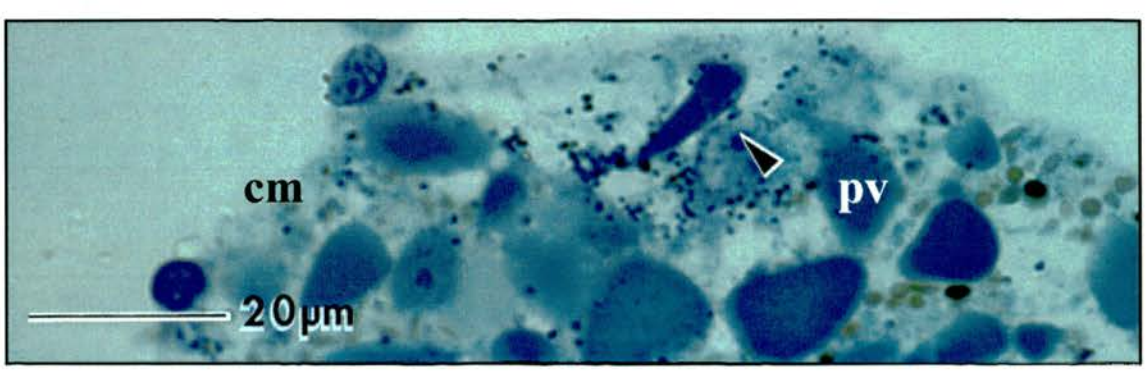
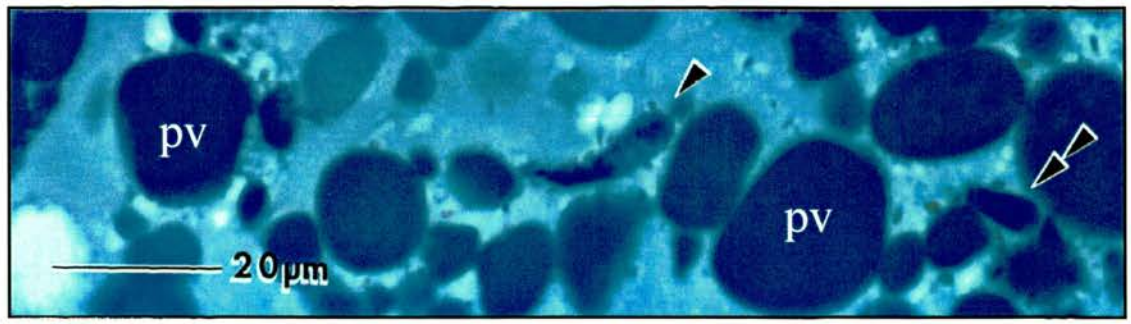
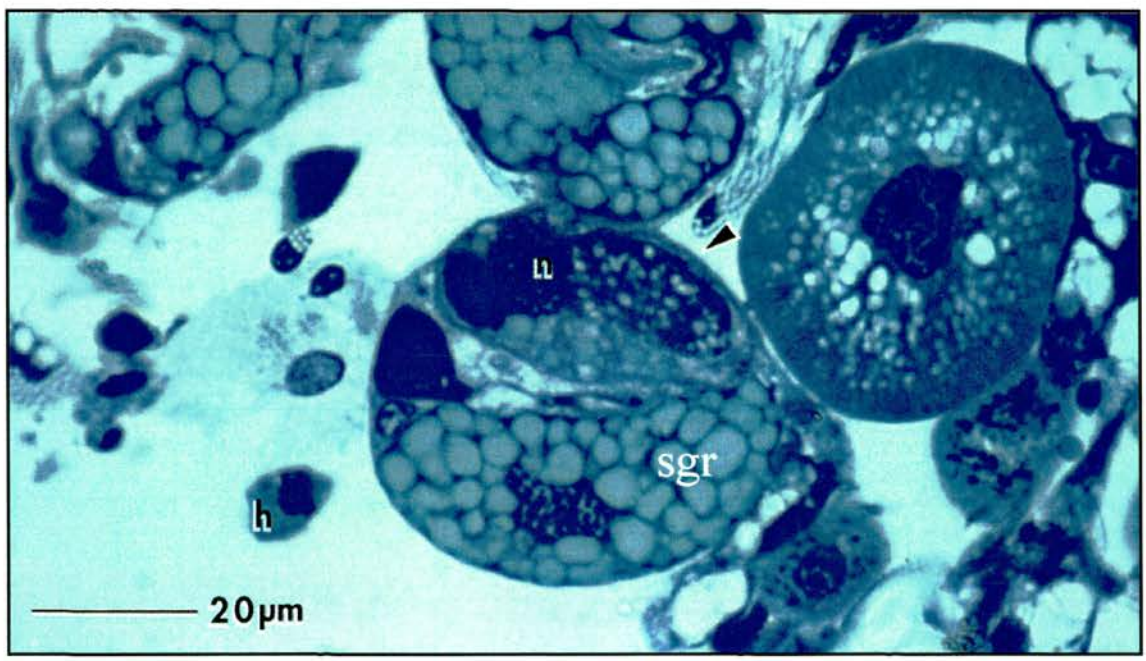

cm
pv
20µm

Figure 5.10m. Kinete in a gut digestive cell in a day 15 post-detachment adult. The arrow shows a kinete in tansverse section, but the double arrow is almost certainly pointing to the anterior end of another kinete. Unless a whole transverse section was displayed, kinetes were not recorded. pv – protein vesicle.

Figure 5.10n. Sporont juxtaposed to the nucleus of an e-cell in a Type III salivary gland acinus in a day 17 post-detachment adult. h - haemocyte, in surrounding haemolymph, n – cell nucleus, sgr – e-cell secretory granules.

Figure 5.10o. Sporoblast adjacent to the hypertrophied nucleus of an e-cell in a Type III salivary gland acinus in a day 17 post-detachment adult. The secretory granules are characteristically reduced compared to uninfected acinar cells. sd - salivary gland duct, h – haemocytes, n – cell nucleus, sgr – e-cell secretory granules.


pv
pv
20µm


n
sgr
h
20µm

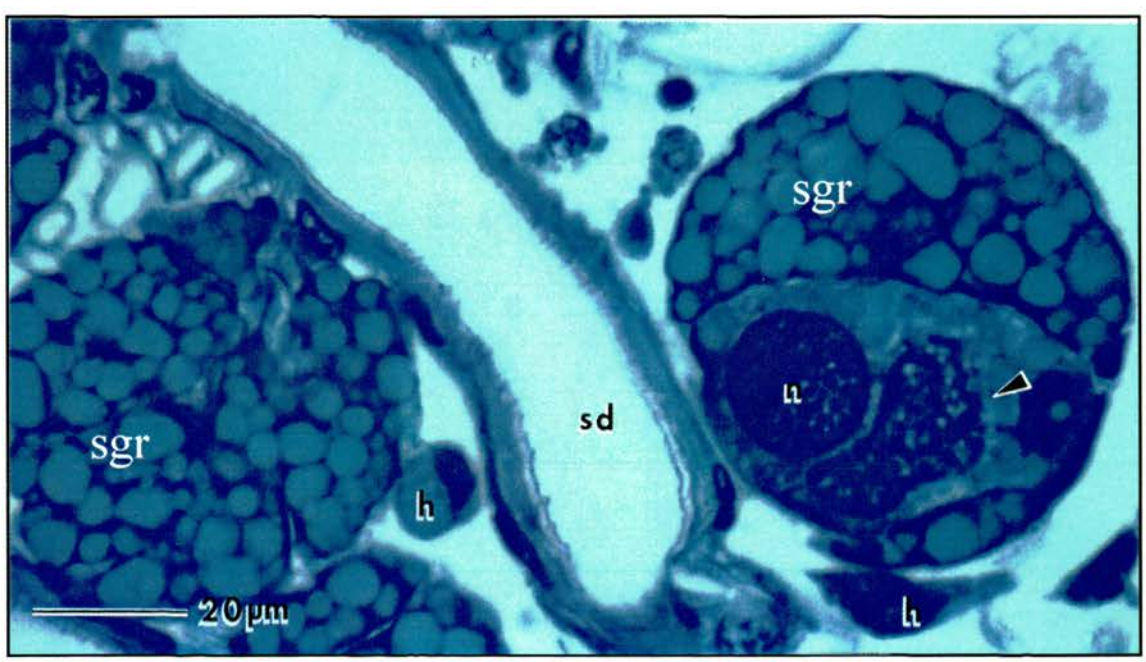
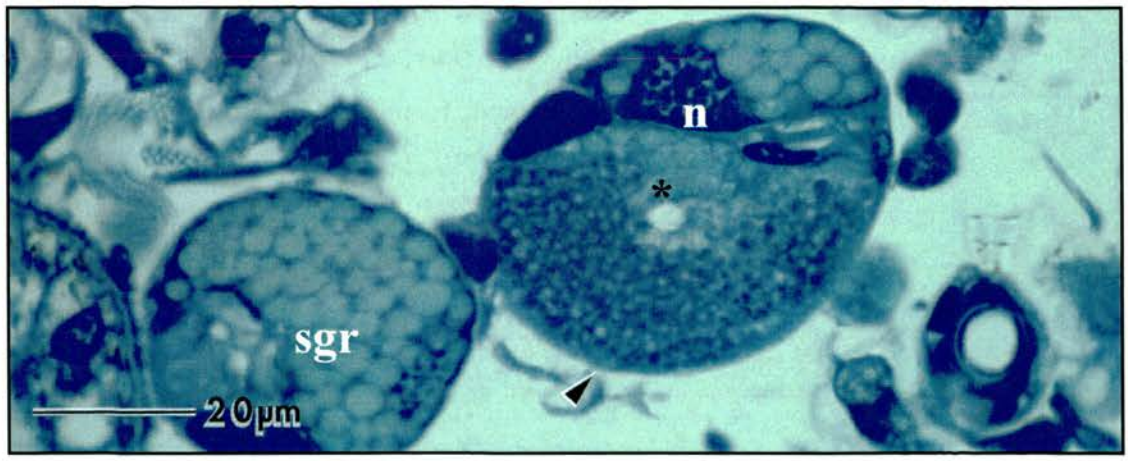
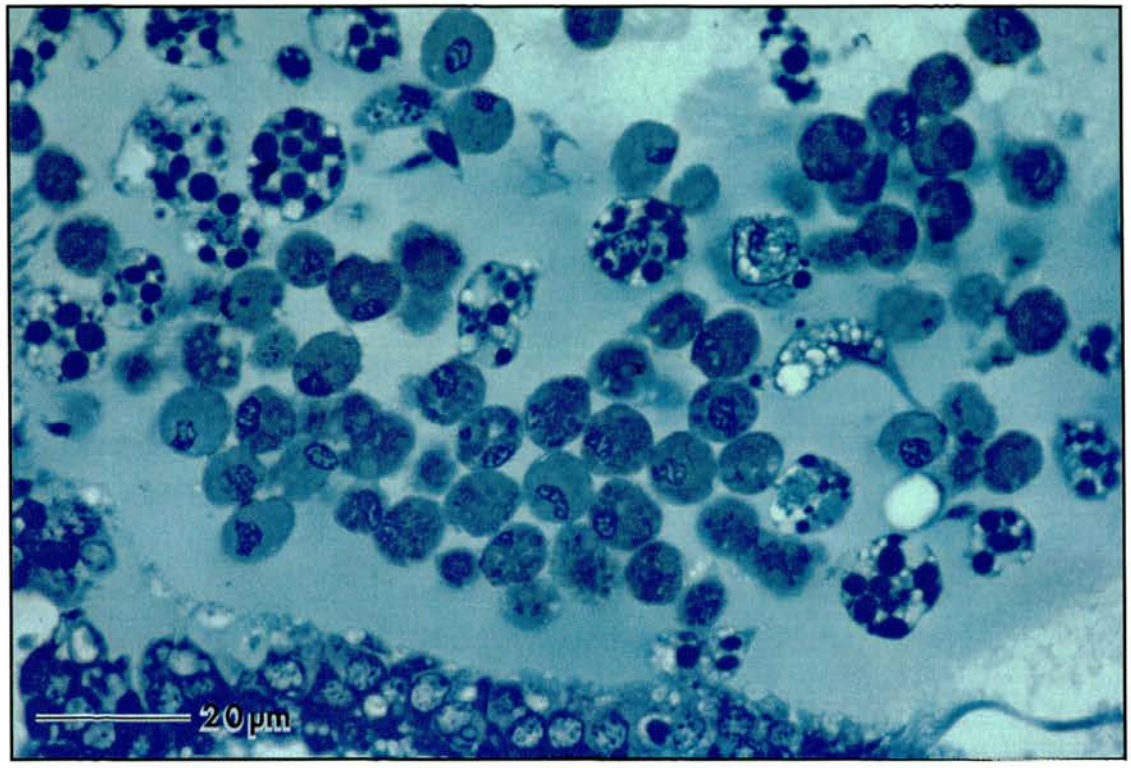

sgr
n
sd
sgr
h
20µm
h

Figure 5.10p. Large sporoblast in a Type III salivary gland acinus in a day 19 post-detachment adult. Secretory granules (*) in the same cell have been considerably reduced in size and pushed to the outside of the cell, as the parasite mass is occupying so much space. n – cell nucleus, sgr – e-cell secretory granules.

Figure 5.11. Haemocytes in sectioned haemolymph.

Figure 5.12a. Zygotes possibly showing pathological effects in a gut digestive cell in a day 16 post-detachment adult. The arrowheads indicate two zygotes showing abnormal colouration. The surrounding protein vesicles are being actively digested (dp) as compared to the more intensely basophilic protein vesicles (double arrowhead) and it appears as though the zygotes may also be partially digested. pv – protein vesicles, rb – residual bodies.


n
*
sgr
20µm


20µm

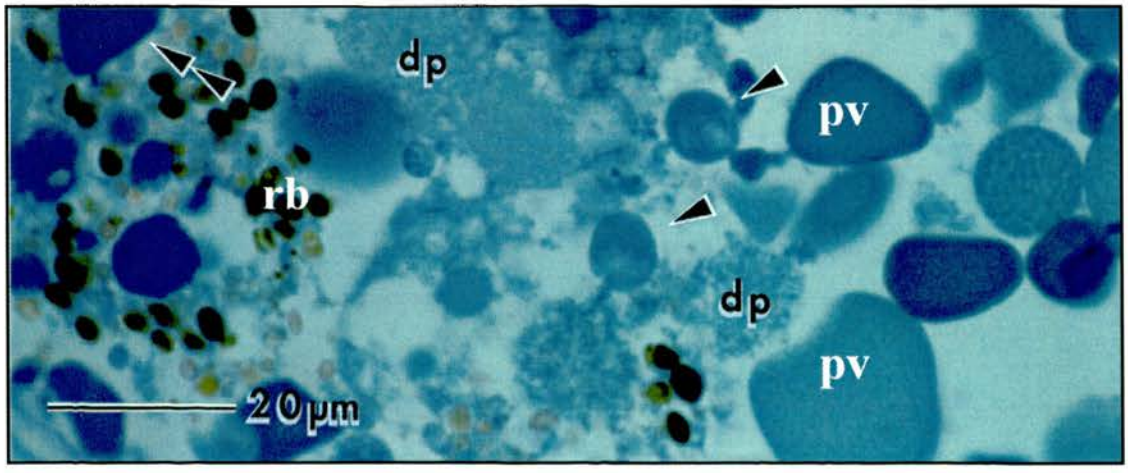
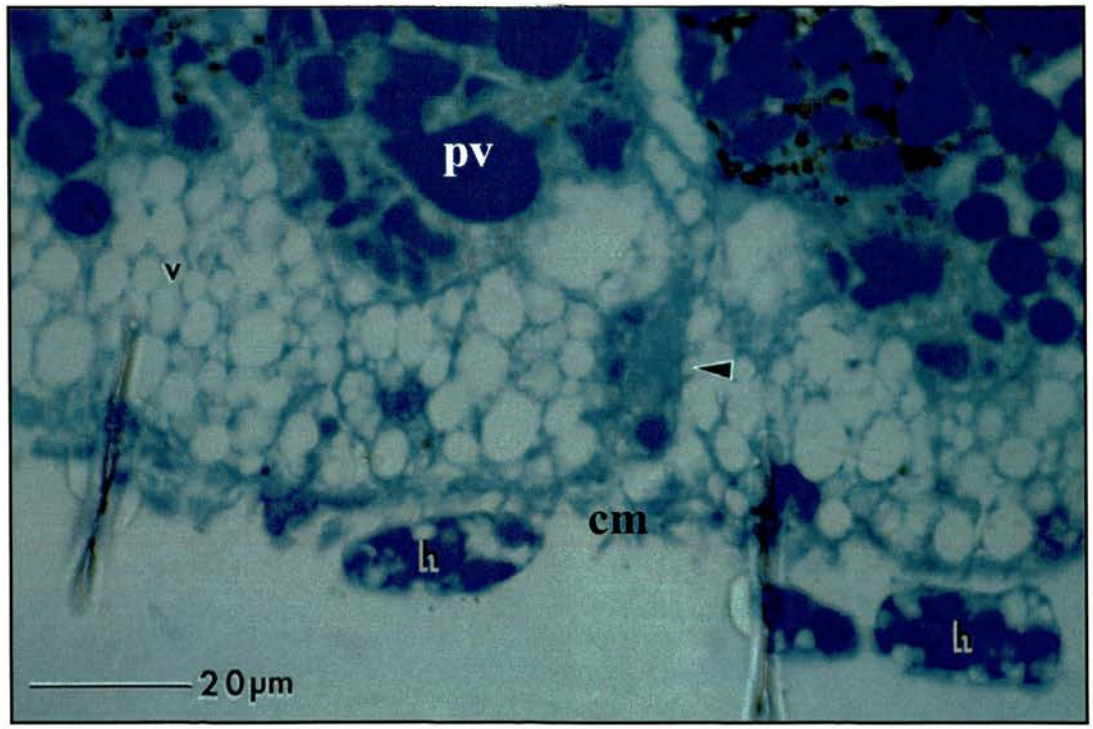
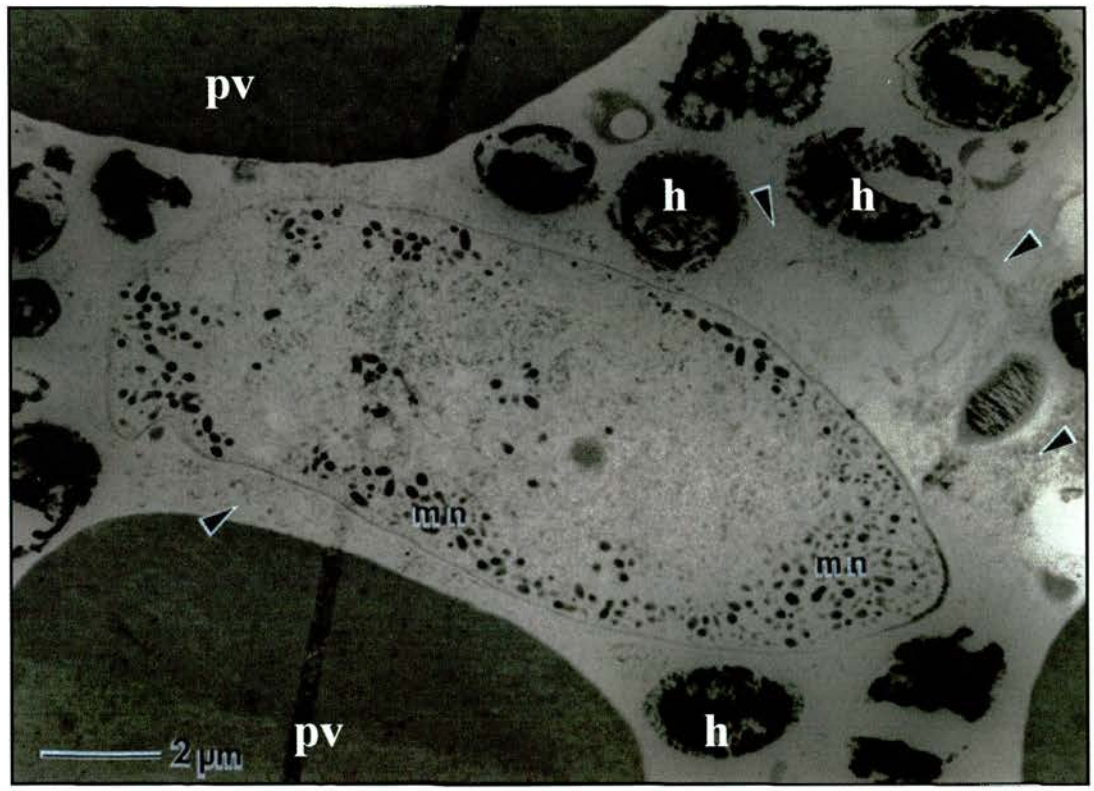

dp
pv
rb
dp
pv
20µm

Figure 5.12.b. A kinete possibly showing pathological effects in a gut digestive cell in a day 16 post-detachment adult. The colour and shape are very indicative of a kinete and small, surrounding protein vesicles appear to be being digested along with the parasite. h – haemocytes, v – vacuoles, pv – protein vaseicles, cm - cell membrane.

Figure 5.13. Transmission electron micrograph through a kinete. The contents of the parasite appear very disorganised and lack any obvious structure apart from the clearly distinguished micronemes (mn). The amorphous material around the periphery of the kinete (arrowheads) may indicate a tick mediated defence mechanism as they were not seen in any other area of the gut. pv – protein vesicles, h – haematin.


pv
v
cm
h
h
20µm


pv
h
h
m.n
m.n
pv
h
2µm

this method, but Fig 5.13 shows a section through a kinete. Although the outer membrane seemed to be intact, and numerous micronemes were present around the periphery of the parasite as described previously (Mehlhorn *et al* 1978), the parasite did appear to show signs of pathology. The cell contents were generally disorganised and amorphous, and no endoplasmic reticulum or internal membranes can be distinguished. The parasite's outer membrane appeared to be surrounded by an amorphous layer, very similar to what Vernick *et al* (1995) described as a 'fuzzy zone'.

Table 5.2 shows the number of *T. parva* forms counted at the various points throughout the tick moult. The raw data for the adult tick infections and parasite forms counted in the histological sections is displayed in Table 3D and 3E, Appendix 3 respectively.

5.6.c. *PCR detection of* T. parva *in the tick gut and faeces*

The PCR results are displayed in Fig. 5.14a - e. Guts from day 0 ticks produced strong amplicons, which decreased in number and intensity by day 5. There is little variation on day 0 as would be expected, but variation is quite apparent by day 5. The level of DNA amplified is very low on day 7 as well but increases very slightly by day 12, to drop again to a low level by day 16 onwards. Day 26 shows one unusually strong band that is difficult to interpret. Amplification from the salivary glands began to occur at day 16 and the amplicons increased in intensity up to day 26. By day 20, uninfected and infected ticks were clearly demarcated, as the amplicons were very intense in the infected ticks and completely absent from the uninfected ones. The DNA was amplified from all three samples of tick faeces. The band was very strong at 0.01g and less so at 0.001g. It was faint, but still discernible at 0.0001g (data not shown).

5.6.d. *Examining haemolymph smears to check for kinetes*

No kinetes were detected in any of the 46 haemolymph smears examined.

Table 5.2. The number of *Theileria parva* forms counted in sections of *Rhipicephalus appendiculatus* throughout the tick moult. The table shows the results of these counts from newly detached nymphs at day 0 through to fully developed adults at day 22. piro % is the piroplasm parasitaemia, n is the number of ticks sectioned and examined. * - these ticks showed abnormalities by day 13 and all died by day 17.

day	piro %	n	mean no. of sexual forms (range)	mean no. of zygotes (range)	mean no. of kinetes (range)	mean no. of sporonts (range)	mean no. of sporoblasts (range)
0	19.6 -22.4*	9	168 (16 - 415)				
2	19.6 - 22.4*	13	18 (1 - 71)				
8	19.6 - 22.4*	13		0.7 (0 - 4)			
9	19.6 - 22.4*	10		76 (3 - 217)	2.8 (0 - 13)		
12	19.6 - 22.4*	9		5.6 (3 - 10)			
13	22.4 - 22.4	15		1.1 (0 - 10)			
15	13.1 - 19.6	14		20 (0 - 66)	0.4 (0 - 3)		
16	19.6 - 22.4*	10		0.5 (0 - 2)	0.1 (0 - 1)		
17	13.1 - 19.6	10		6.3 (0 - 50)	0.1 (0 - 1)	1.3 (0 - 7)	
19	13.1 - 19.6	7		0.3 (0 - 1)		3.0 (0 - 11)	0.1 (0 - 1)
21	13.1 - 19.6	12		0.1 (0 - 1)		0.3 (0 - 2)	0.4 (0 - 2)

Figure 5.14a - e. Gels showing PCR results for *Theileria parva* detection in different tick organs throughout the moulting period. Results from 10 ticks in each group are shown. **a**. Guts from day 0 post detachment showing a fairly uniform level of *T. parva* within them; **b**. Guts from day 5 post detachment, showing a great reduction in parasite burden and individual variation becoming more prominent; **c**. Guts from day 26 post detachment show *T. parva* detection in only 2 guts; **d**. Salivary glands from day 22 post-detachment; **e**. Salivary glands from day 26 post detachment showing very intense amplicons. The arrowheads point to the expected amplicon size of 405bp. *- represents overspill of molecular weight marker due to a damaged well.

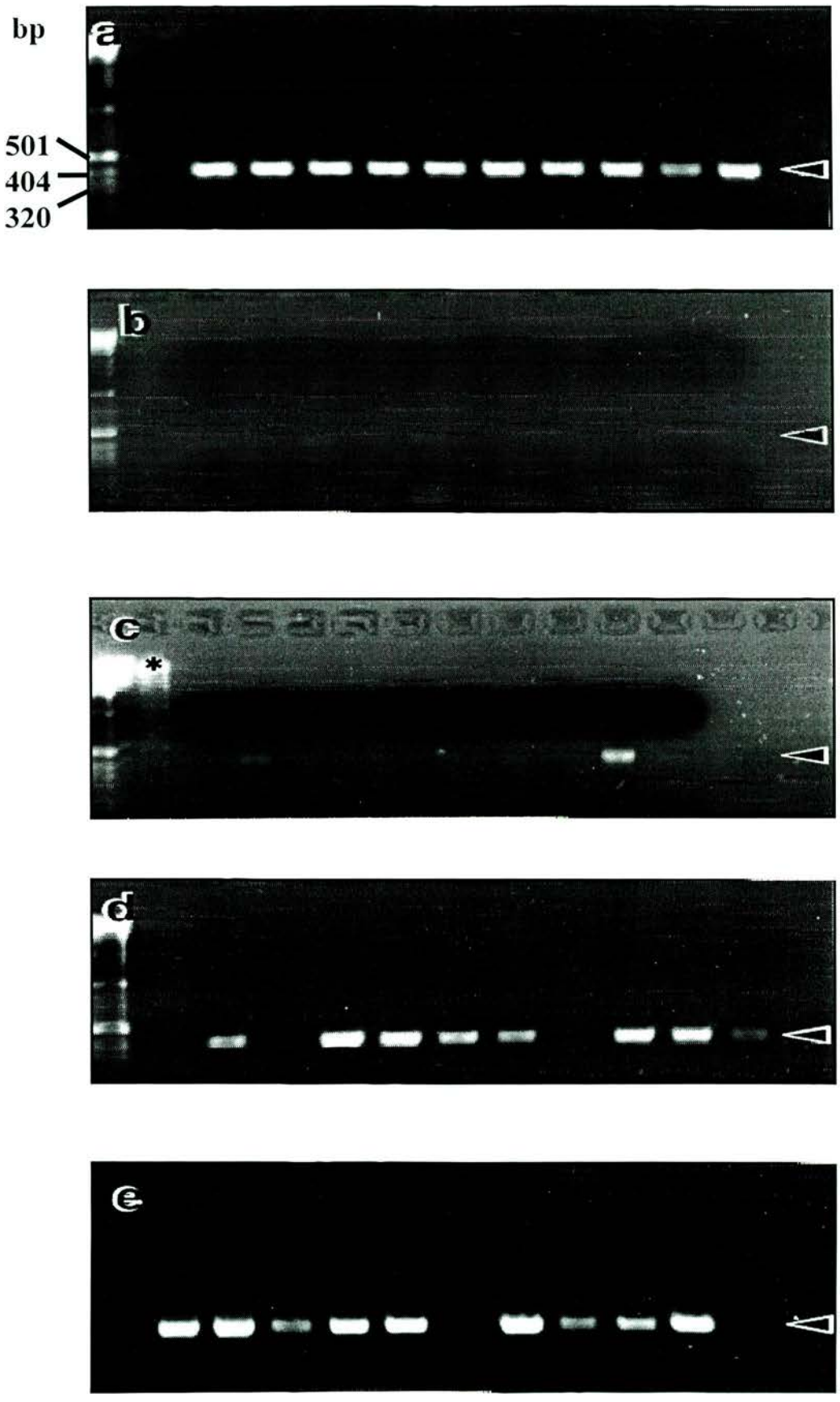
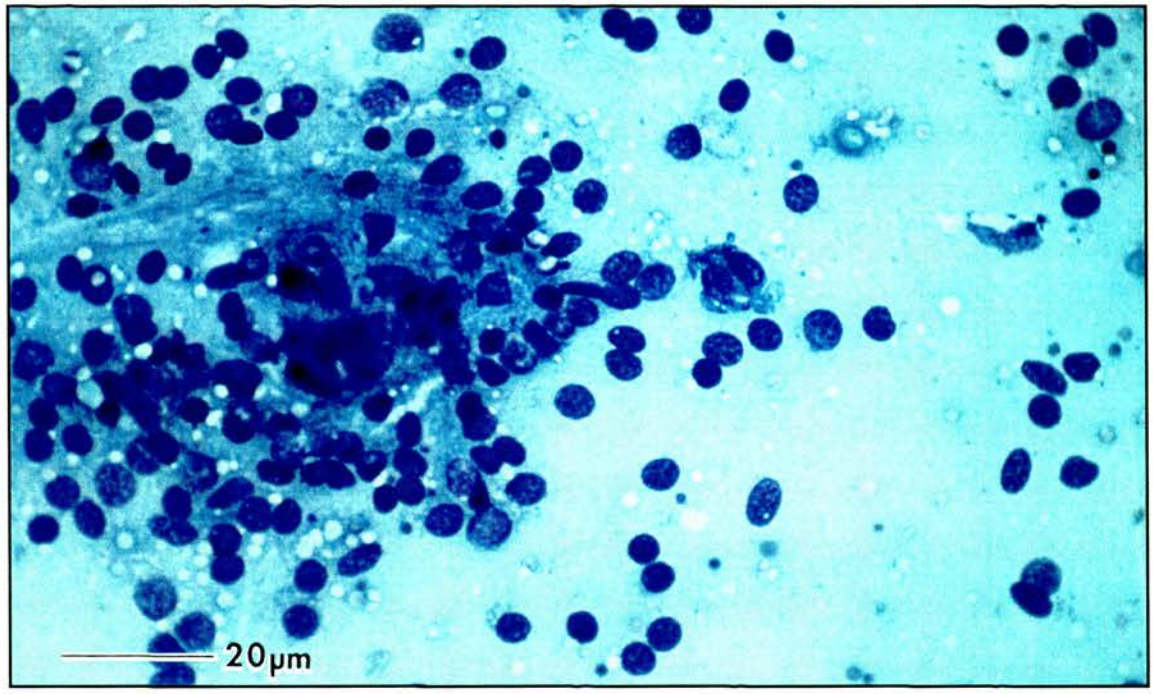
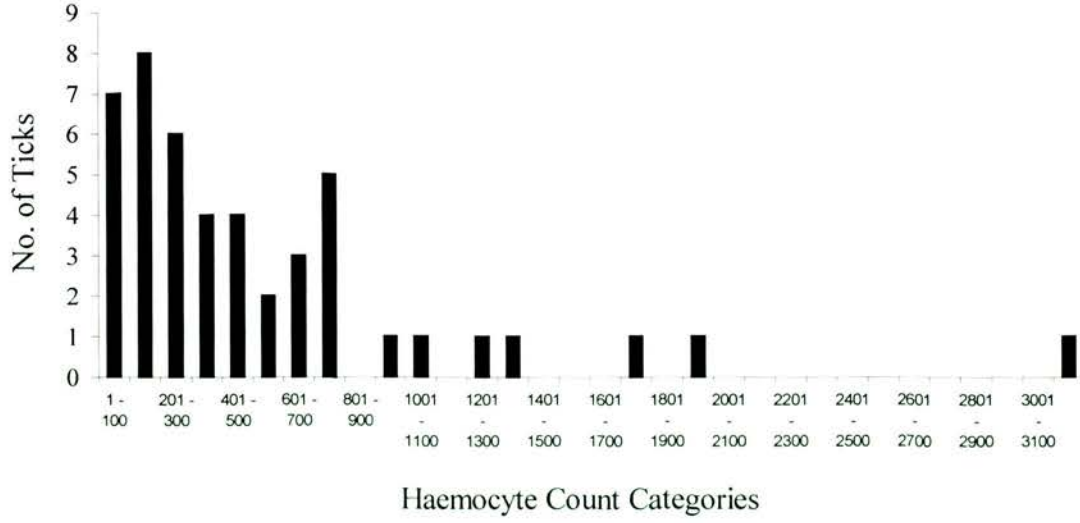

bp
501
404
320
a
b
c
*
d
e

Figure 5.15. Haemolymph smear from a day 16 post-detachment adult.

Figure 5.16. Graph showing a frequency distribution of haemocyte numbers from smears. n = 46.


20µm


No. of Ticks
0
1
2
3
4
5
6
7
8
9
1 - 100
201 - 300
401 - 500
601 - 700
801 - 900
1001 - 1100
1201 - 1300
1401 - 1500
1601 - 1700
1801 - 1900
2001 - 2100
2201 - 2300
2401 - 2500
2601 - 2700
2801 - 2900
3001 - 3100
Haemocyte Count Categories

5.6.c. *Haemocyte counts*

Only 46 out of the 50 smears made were examined and counted, as four were rejected on the basis of gut content contamination. Fig. 5.15 shows a micrograph of a typical haemolymph smear. An overdispersed distribution of haemocytes occurred with 1 to 800 haemocytes counted in smears from 85% of the ticks, while the other 15% had counts ranging from 900 to 3100. Fig. 5.16 shows the frequency distribution of haemocyte numbers from day 16 ticks.

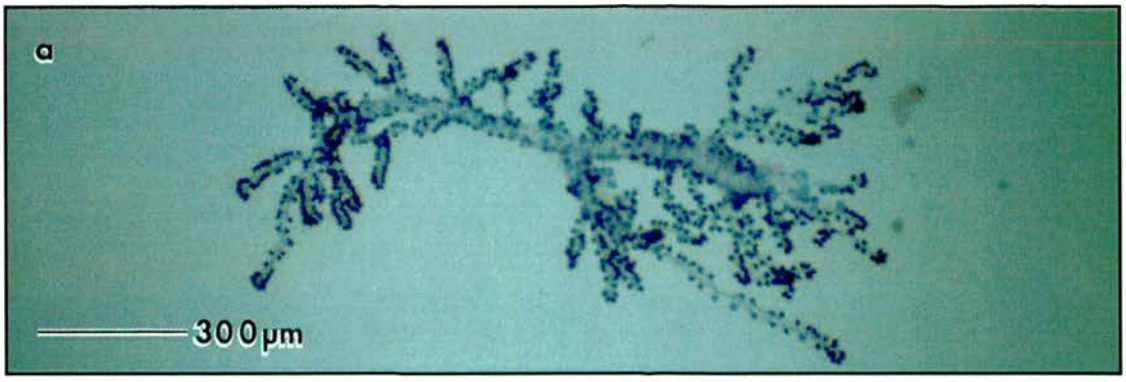
5.6.f. *Examining the structure of developing salivary glands*

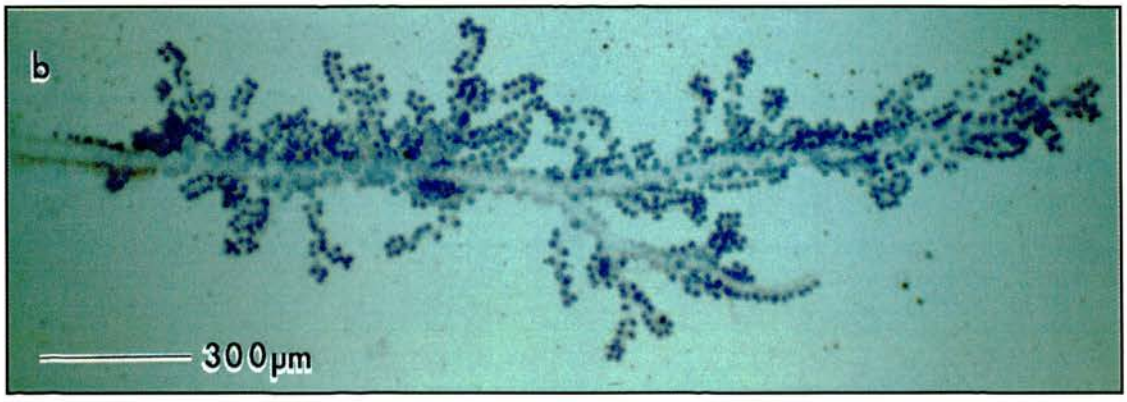
The salivary glands in the ticks regressed to such an extent by day 4 post detachment that only the main duct could be seen. The subsidiary ducts and acini could no longer be distinguished. They grew slowly until day 12, when the number of ducts and tiny undeveloped acini began to increase in number. The duct structure was better developed by day 14 and the acini had grown bigger than at day 12. The fastest rate of development occurred from day 14 to 16. By this stage the glands looked like immature versions of the eventual adult form. The acini were still undeveloped with largely undifferentiated cell types, but by day 18 the glands had grown to almost full maturity. Figs. 5.17a - d shows the MGP stained glands from day 12 to 18. As sporonts were seen in the histological sections of salivary glands by day 17, it is likely the kinetes penetrate the glands around day 15/16 (Fig 5.17c).

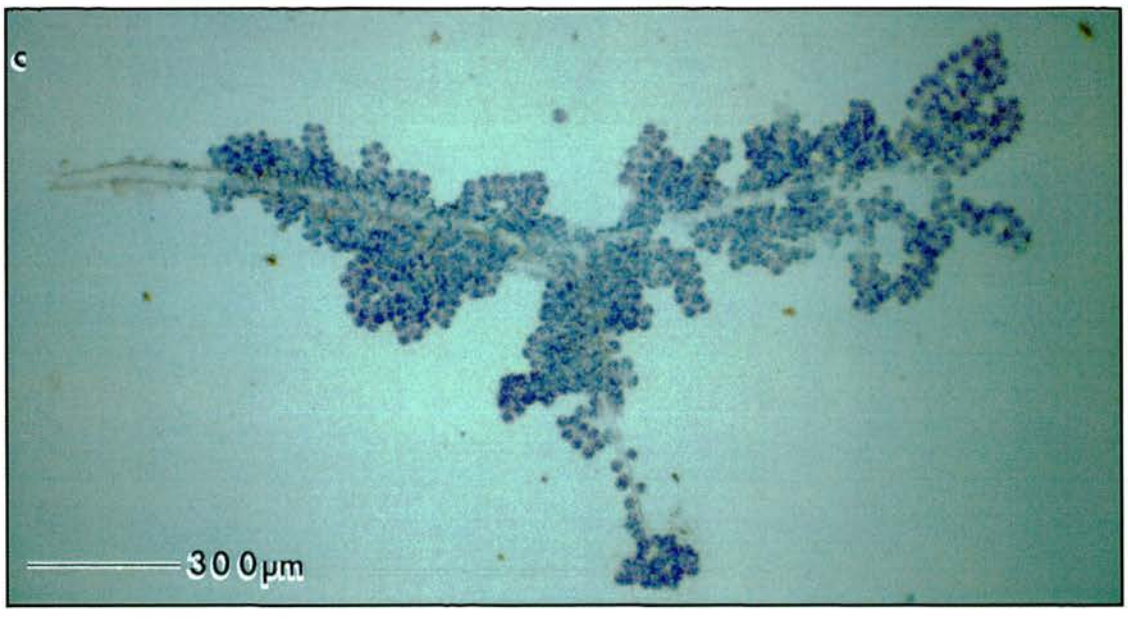
Discussion

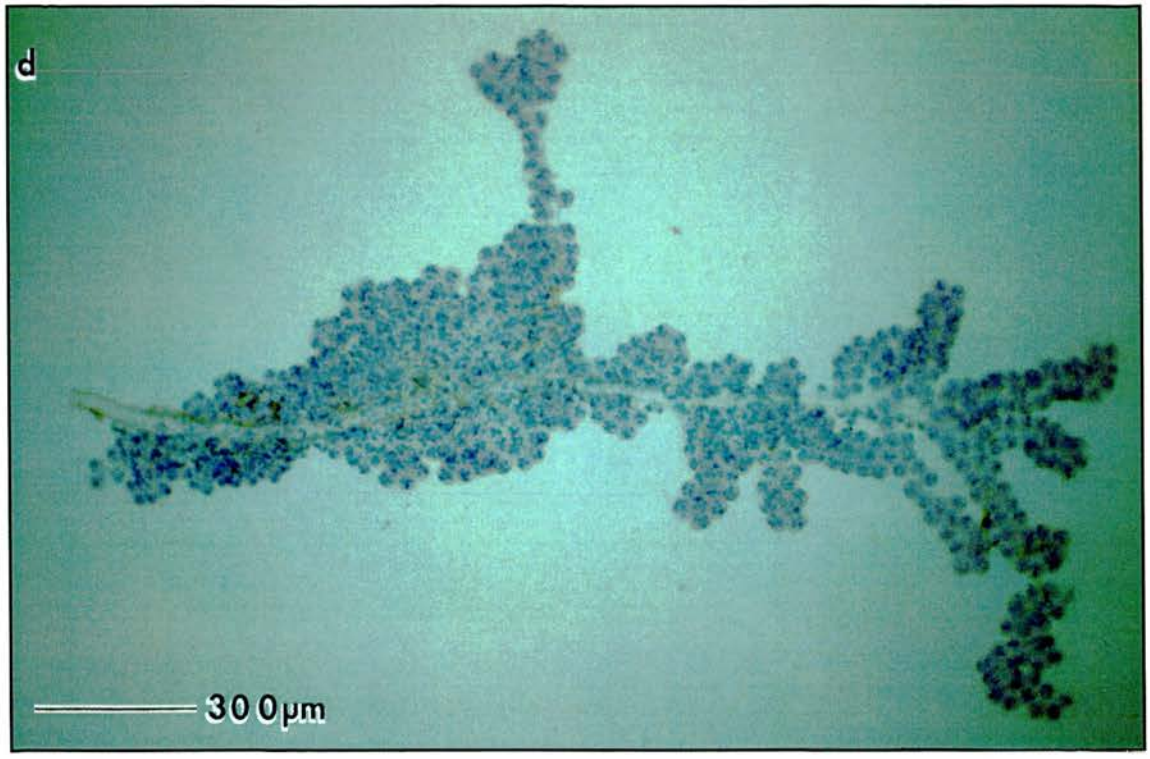
The development of *T. parva* forms is intimately involved with that of *R. appendiculatus*. It is common parlance to refer to 'windows of opportunity' with regard to this situation. Gametes have a short period in which they must fertilise and then the zygote must penetrate the gut wall or they will be digested in the lumen or passed out of the tick with the excreta. Zygotes have a specific time period in which they must develop within the digestive cells (presumably influenced by the ticks' stage of digestion) and turn into kinetes. One of the main obstacles the parasites will face at this stage is likely to be the production of acid phosphatase into the gut by the secretory cells (Walker and Fletcher 1987). Kinetes have a specific time

Figure 5.17a-d. Salivary glands dissected and MGP stained at various intervals throughout the nymph to adult moult. **a**. day 12; **b**. day 14; **c**. day 16; **d** (overleaf). day 18.


a
300µm


b
300µm


c
300µm



period in which they can penetrate out of the gut epithelium and enter the salivary glands and finally, sporonts have a specific time period in which they have to develop into sporoblasts and then produce sporozoites to coincide with salivation at the next tick feed. Results from the first experiment that involved different incubation temperatures agreed with much of what has been previously published. The ratio of moulting time and post moult development with decreasing temperature, i.e. moulting time is doubled for a 5^0C drop in temperature, seems to be a consistent characteristic which has been observed in all *R. appendiculatus* stocks and strains examined to date. A standard moulting temperature of 28^0C is used in this laboratory because the moulting and post moult development time of one month is convenient for practical reasons, and high levels of *T. parva* infections occur in our hands. Young and Leitch (1981a) obtained the highest *T. parva* (Muguga) infections in *R. appendiculatus* (Muguga) when they were incubated at 28^0C and significantly less at 23^0C (prevalence reduced by 30% and abundance reduced by 64% at 23^0C), but another set of experiments using the same parasite and tick strains (Young and Leitch

1981b) showed that a 23°C incubation temperature produced higher infections than 28°C (prevalence reduced by 12% and abundance reduced by 60% at 28°C). Young and Leitch (1981a) reported that zygotes and kinetes are present only very briefly in the gut of ticks incubated at 28°C, considerably longer at 23°C and for over 70 days at 18°C. The kinetes seemed unable to progress past this point at 18°C and did not go on to form sporoblast infections. Young and Leitch (1981b) showed that large numbers of gametes developed in the gut of nymphs exposed to quasi-natural conditions (25 - 7°C diurnal temperature fluctuation) and persisted until day 20 post-engorgement. This contrasted with the low numbers of sexual forms detected in ticks incubated at 28°C which persisted only until day 5 post-engorgement. It was suggested that the lower metabolic rate of the nymphs under the former conditions is suitable for the development and persistence of gametes. Describing *Babesia* development in the gut of *Boophilus* ticks, Agbede *et al* (1984) suggested that a form of sexual fusion takes place resulting in a zygote which is spherical and motile. Although parasites do not develop through the tick moult at 18°C, sporonts survive for extended periods of time in the salivary glands at this temperature. The sporont is a quiescent form adapted to the prolonged questing period of nymphs and adults of *R. appendiculatus*. It is possible that developing forms, that may be in the process of reorganising their metabolic activity or outer membrane characteristics, are more vulnerable to tick mediated destruction than the sporonts. Their motility may be compromised to such an extent by the lower temperature that they are unable to get out of the cell in time. Literature on the metabolic differences between the arthropod stages of *Theileria* is virtually non existent. Mehlhorn *et al* (1978) reported that they were unable to find micropores or larger cytostomes in zygotes whereas they were present in the erythrocytic and mature kinete stages, the relevance being that they could not understand the means by which zygotes would be able to consume enough nutrients to sustain their huge growth. It may be that sporoblast survival at this temperature is possible because the salivary glands are a relatively immunologically privileged site compared to the gut and haemolymph compartments.

Results of the study comparing the batches of adult ticks that had been exposed to *T. parva* once and twice in previous instars showed stark differences in that the twice infected ticks were significantly less infected than the once infected ones. However, they were possibly confounded by lack of infection within the salivary glands of Batch A nymphs (Batch A ticks were infected with *T. parva* as larvae and nymphs, whereas Batch B were only infected as nymphs). The larvae had engorged at high piroplasm parasitaemias, and nymphs detaching on the same day moulted through to very highly infected adults. Salivary glands of nymphs are much smaller than adult glands. Ochanda *et al* (1996) reported a mean number of 127 acini in nymphs compared to 2164 and 2233 in males and females respectively. Thus they have a smaller capacity for numbers of sporoblast infections in comparison with adult ticks. However, it has been established that nymphs can transmit the disease. Ochanda *et al* (1996) showed nymphal infections where the abundance values were very low compared to that of adults, but the prevalences were only slightly less, using *T. parva* Muguga in *R. appendiculatus* Muguga. Infections were demonstrated in nymphs that developed from larvae that had engorged on lower piroplasm parasitaemias than the ticks in the experiments described here fed on. Purnell (1974) considered making stabilate from nymphs because he found higher infection prevalences per unit weight of tick with them, and greater ease of grinding them for GUTS stabilate production, than for adult ticks.

The difference in mass between engorging nymphs from Batch A and Batch B was very interesting. The batch of previously infected ticks fed to a significantly lower weight than the ticks that had previously not encountered *T. parva* (having been rabbit fed as larvae). It would have been preferable to have fed the control batch (Batch B) on an uninfected calf, but this was not possible due to cost. However, in their very comprehensive investigation into factors affecting the outcome of infection with *T. parva* in *R. appendiculatus*, Büscher and Tangus (1986) showed no influence of larvae feeding on bovine or rabbit hosts on the resulting adult infections after the nymphs had fed on the same infected calf; therefore it seems unlikely that the difference in engorged weight between the two batches was due to host differences.

Infection was not demonstrated in the salivary glands of Batch A nymphs. The glands looked healthy and free from any obvious signs of damage that could have been caused by invading kinetes or developing sporoblasts that may subsequently have been destroyed. It is probable therefore, that the lower engorgement weights seen in Batch A nymphs (exposed to *T. parva* infection as larvae) were caused by the inability of their guts to expand sufficiently to allow as large a bloodmeal as Batch B nymphs (larvae were uninfected) could consume. This is in contrast to a possible inability to feed properly due to salivary gland malfunction as a result of infection from the larval feed. The inability of guts of Batch A ticks to ingest an equivalent amount of blood to Batch B may have been caused by pathological effects of zygote or kinete activity within the gut (see Chapter 6). The lower sporoblast infection levels in Batch A adults compared to Batch B cannot be accounted for by the reduced number of piroplasms ingested in the smaller bloodmeal. Büscher and Tangus (1986) found no correlation between nymphal weight on resulting adult infection and Ochanda *et al* (1996) commented that the reduced bloodmeal volume of larvae as opposed to nymphs would only make an impact on infection (all other things being equal) in the next instar if the parasitaemia was very low. At high parasitaemias, there are such an excess of piroplasms and sexual stages relative even to a high sporoblast infection, that a reduction in numbers by half or a quarter would have no impact on the eventual infection.

There are mixed reports regarding the susceptibility of male and female ticks to infection. Büscher and Tangus (1986) reported that male and female ticks probably vary in their susceptibility to different strains of *T. parva*. Ochanda *et al* (1996) showed slightly higher prevalence, and considerably higher abundance values in females compared with males, but the sporoblast infection results presented throughout this thesis and from many other sources indicate that differences between the infections of males and females tend to be non-significant. The females in Batch A however, were significantly less infected than the males. This may indicate that female ticks are capable of mounting a more effective acquired immune response to *T. parva* than males.

A very obvious immune effect noted was the presence of what may be polyphenoloxidase enzyme action on the glands of the ticks that fed on calf 404. Batch A ticks had a significantly higher level of the deposit than did those from Batch B. Most of the glands that displayed the deposit were uninfected suggesting that the phenoloxidase may have been one of the immune factors involved in limiting the infection within Batch A ticks. If so, it would only be part of the immune repertoire as the effect was only noted on a relatively small proportion of the total glands. The effect of leakage of gut contents noted in the ticks which fed on calf 14500 was not significantly different between Batch A and Batch B and therefore could not be attributed to an acquired immune response of any kind. It may have been caused by damage from kinete migration, especially as it was concentrated over the area of the salivary glands. One of the major points the two effects demonstrate is the difference that can result from individual host animals.

There are no data available on actual nymphal infection levels in the field from salivary gland examination. This is because of the greater difficulty of collecting and dissecting nymphs compared to adults. Norval (1989) reported that around 94% of clinical ECf cases in Zimbabwe occur around January to March, a period in which mainly adult ticks feed. However, this is not a suitable index on which to judge whether nymphs carry infection or not. Because of the lower parasite abundances harboured by nymphs it has been suggested that nymphs may be very important in delivering low doses of *T. parva* into cattle, thereby inducing immunity with no apparent clinical signs (Ochanda *et al* 1996). Koch *et al* (1993) stated that nymphal transmission of *T. parva* bovis in Zimbabwe was previously assumed either not to occur, or to be insignificant because very few cases of disease occurred during and immediately after the time of year they fed. However, in experimental infections it was shown that *R. appendiculatus* nymphs were capable of transmitting serious clinical disease to susceptible cattle after the larvae had fed on infected cattle.

Areas in which live *T. parva* vaccine is administered are likely to have high proportions of carrier-state cattle, which will expose ticks to more infection than at

present when disease control measures usually consist of regular acaricide usage. It appears that the nymphs do not have to carry salivary gland infections themselves to allow an anti-*T. parva* immunity to develop in the adult stage. It is possible that the low level parasitaemias present in carrier animals which could be taken up by engorging larvae may be sufficient to trigger an immune response within the adult stage of the tick, if a nymph had also engorged on a carrier animal. This will be important work to carry out in the future. Feeding larvae on hosts with very low parasitaemias, representative of a more typical field situation may reveal whether the same level of immunity, if any, develops.

It is important to stress that the numbers of any one parasite form counted in the histological sections cannot be related to another in a direct, quantitative way. The macrogametes are about 4-5μm in diameter, zygotes grow from 7μm to 12μm, kinetes measure 5.5μm x 19μm and the sporoblasts are larger still at anything up to the diameter of a Type III acinus (~100μm) depending on their state of maturity. Because the sections were 1 to 2 μm thick, counting 100 sexual forms in one section could easily mean that 100 *other* gametes could be present in a section cut 4 to 8 micrometers further into the gut lumen, indicating the lumen could contain many thousands of gametes. Because of the larger size of zygotes however, 100 seen in one histological section would very probably also be present in another section cut 2 to 4 μm further into the gut lumen. This principle will be even more true of the sporoblasts as they are larger still. This simply indicates that there will be significantly lower numbers of the larger parasites present even if a single count from one section seems quite similar.

The difference in the number of gametes seen on days 0 and 2 is more likely to be a reflection of the physiological state of the tick gut than a true indication of parasite numbers. As the protein became incorporated within vesicles, the gut became very densely packed and the sections extremely basophilic which made examination for gametes very difficult. At day 4, the lumenal spaces had disappeared completely

from all of the ticks' guts and were tightly packed with motile digestive cells. No parasites were seen on days 4 and 6, possibly as a result of this.

Zygotes were first seen on day 8. The data for zygotes show a bimodal distribution with the first peak at day 9 and the second on day 15. A possible explanation for this is that ticks from different detachment days were used for these samples. As can be seen from Table 5.2, the sections examined on days 0 to day 12 (and day 16) were from the tick batch that detached on day 3 of the engorgement period (D3) whereas the samples on day 15 and 17 to 21 were from ticks that dropped on day 4 (D4) and 2 (D2) of engorgement respectively. The first peak of zygotes was very high and coincided with the appearance of kinetes. This is exceptionally early for kinetes to appear and was 3 - 4 days before the ticks would have been due to moult. Young and Leitch (1980) reported that kinetes from three strains of *T. parva* were first detected on days 12 or 13 (one to three days before they moulted) in *R. appendiculatus* (Muguga isolate) moulting from nymphs to adults. Schein *et al* (1977) reported the first appearance of kinetes on days 19 to 20, although the *T. parva* strain they were using appeared to take an unusually long time to develop in their ticks. Some of the zygote numbers were very large here as can be seen in the range data. Zygotes obtain all their nutrition from the gut digestive cells and it is very likely that the ticks at this stage were detrimentally affected by the high density of parasites they were supporting, as was demonstrated in *Hyalomma* ticks infected with *T. annulata* (Schein and Friedhoff 1978). Zygotes in a normal tick/parasite relationship would be seen much later than day 8, probably starting at day 12/13 as evidenced by the second peak (with the least pathologically affected ticks). The number of zygotes dropped markedly by day 12 as did the general health of the D3 ticks that were being used. Details of the tick pathology will be given in the Chapter 6, but it is necessary to mention a few facts here. The moulting seemed to stop in the D3 ticks (as compared to the ticks moulting in the control batch and others from different detachment days from the same calf) and their digestion seemed to have been greatly slowed. All D3 ticks died by day 17. This early peak of zygotes and kinetes may represent an artifact caused by a breakdown in the normal relationship between *T.*

parva and *R. appendiculatus*. For some unknown reason, the zygotes seem to have developed too soon and in higher numbers than the ticks seemed able to sustain. If the kinetes had migrated from the digestive cells to the salivary glands on days 10 or 11, it is very unlikely they would have been able to complete their lifecycle because the primordial salivary glands would not have developed to the point of allowing kinete penetration and survival. The large drop in zygote numbers, and total loss of kinetes at day 12 is probably a result of parasite and tick death caused by their premature appearance by day 8/9. This peak will probably not represent the usual timing of events that would take place between the parasite and vector. The second peak of zygotes and kinetes on day 15 is much more representative of normality. Young and Leitch (1980) showed the **first** appearance of *T. parva* Muguga kinetes in the gut of *R..appendiculatus* Muguga on day 12/13, which would tend to support the evidence presented here as it is likely their numbers would increase before migrating from the gut to the salivary glands.

Of interest is the rapidity with which the numbers of zygotes and kinetes rise and fall. In the first peak, the numbers rise very quickly from day 8 (mean. 0.7 zygotes, 0 kinetes) to the day 9 level (mean. 76 zygotes, 2.8 kinetes) and then down again to day 12 (av. 5.6 zygotes, 0 kinetes). The second peak shows a rise from day 13 (mean 1.1 zygotes, 0 kinetes) to day 15 (mean. 20 zygotes, 0.4 kinetes) and then down to the day 17 level (mean. 6.3 zygotes, 0.1 kinetes). The first appearance of sporonts was on day 17. As the parasite does not actually multiply in the tick until it reaches the salivary gland, the reason that the zygotes appear to increase in number is that they simply become detectable. Their rapid appearance in high numbers is possibly indicative of a survival mechanism. The digestive cells may be a hostile environment for the parasites when they are at the mature zygote to kinete stage. This indicates that it may be just as critical for the zygote to mature rapidly into a kinete as it is for the kinete to migrate from the gut to the salivary gland.

Young and Leitch (1981a) found that zygotes and kinetes developed as normal in the gut of ticks incubated at 23^{0}C and 28^{0}C and were also present in those incubated at 18^{0}C. In the 18^{0}C incubated ticks the kinetes did not develop any further suggesting the gut was a major barrier. The lack of kinete motility due to the lowered temperature or the fact that the physiological processes which link the bloodmeal digestion to the salivary gland development were proceeding too slowly, and kept the parasites in the gut for too long, may have been reponsible for the parasites not developing past the gut at 18^{0}C.

If a zygote develops within a digestive cell, which then buds off from the gut lining to become motile, as opposed to one which remains sessile, it will presumably have a reduced chance of survival (*e.g.* Fig. 5.10i). The parasite in the example referred to will have to penetrate the motile digestive cell just to emerge into the gut (as opposed to the haemolymph). Even if the gut does not represent a chemically hostile environment for a kinete, at the very least, this situation will result in the kinete having to spend considerably more time in the gut than one which will penetrate the salivary glands directly from the epithelial cells. This increased time it will take to leave the gut could allow the salivary glands to develop past the point of being susceptible to infection by the kinetes.

One of the ticks in the day 17 sections had 50 zygotes within it (see Table 5.2, range data) which was very high by this stage - as early sporoblasts were seen in the salivary glands of other day 17 ticks. The bloodmeal digestion in this tick was particularly slow and this was reflected in the development in other internal organs. The salivary glands and the number of zygotes (as compared with other ticks at the same stage) looked as if they belonged to a day 15 tick. This emphasizes the importance of the tick physiology on parasite development, or possibly the correlation of parasite development on tick physiology.

There was a large variation in the sporoblast development seen in salivary glands. Variation was demonstrated earlier with the maturity of sporoblasts in the MGP

stained salivary glands from ticks incubated for 5 days at 37^{0}C. This variation may be a consequence of the sporoblasts' starting point, before any conditions are encountered which would cause salivary gland and sporoblast activation. Purnell and Joyner (1968) and Ochanda *et al* (1988) showed that the sporoblasts from some ticks were infective to cattle after only one day of feeding as opposed to the majority which require 3 to 4 days feeding before they become mature enough to cause infection.

The nymphal blood volume data is useful for making more accurate estimates of the initial level of piroplasm ingestion than has been available previously (Purnell 1974). It should be noted that the technique used was flawed in that it assumed the blood of all animals tested would have equivalent PCV values (and therefore equivalent haemoglobin contents). A more accurate method would have been to use measured volumes of pure haemoglobin bought from a commercial supplier to produce a standard curve. The advantage of actually measuring the level of blood ingestion though, is that a figure can be quoted relative to the size of the tick. Table 5.3 shows the level of piroplasm ingestion relative to the sporoblast infection levels that resulted.

Table 5.3. Piroplasm levels in the bovine blood, nymph gut upon engorgement and the resulting infection levels in the adult tick. Batch A ticks were exposed to *T. parva* as larvae and nymphs. Batch B and Batch C ticks were exposed to *T. parva* only as nymphs. Preparation of Batch A and Batch B was described in Section 5.2 and Batch C in Section 5.1 (*n.b.* Batch C was only named as such for the purposes of this table).

ticks	calf / piro (%)	mean engorgement weight (mg)	mean piroplasm ingestion	prevalence / abundance
Batch A	404 / 8.0	8.9	1.2×10^7	85 / 63.2
Batch B	404 / 8.0	5.3	7.1×10^6	25 / 1.3
Batch C	691A / 0.4	9.0	5.9×10^5	55 / 12.0

The table shows that the piroplasm ingestion in Batch A nymphs was actually considerably higher than in Batch C nymphs, even though the resulting adult infections were very much lower. This shows that the lower engorgement weights of Batch A ticks were not responsible for the lower infections. It is likely that other factors within the ticks, possibly as a consequence of their previous encounter with *T. parva* as larvae, influenced their resulting infections.

No kinetes were seen in the general plastic matrix that remained on the sections. The dissection used a large volume of saline in which some haemolymph was lost. However, the sections revealed many haemocytes trapped within stained haemolymph between gut caecae and other viscera showing that many haemocytes were retained around the viscera. No interactions between kinetes and haemocytes were seen and no kinetes were seen in the haemolymph smears. Unlike *T. annulata* it is possible that *T. parva* kinetes do not travel through the haemolymph. They may go directly from the basal lamina of the gut to the basal lamina of the salivary glands that are closely packed together. This is supported indirectly by the phenoloxidase enyme effect around the salivary glands in Batch A adult ticks (infected with *T. parva* twice previously). The leaky gut effect (which could have been caused by kinete migration) was concentrated on top of the salivary glands where it was in contact with the gut as well. No direct evidence of kinete penetration of the salivary glands in this manner was provided so this cannot be confirmed as yet. It is unlikely that with the large number of sections examined, kinetes would not have been seen in the haemolymph if they had been present. If the zygote to kinete transformation is a stage vulnerable to immune attack, it may be that the number of kinete penetration events is not much greater than the number of sporoblasts that develop. If this is the case, there would be very few kinetes relative to the area of the gut, and in contact with salivary glands, which would make sectioning and viewing them very difficult. The same situation was envisioned by Purnell *et al* (1974) when they suggested that looking at sections of whole, engorged nymphs would be a suitable method for viewing kinete migration by such direct transfer. Kinetes may have a very small window of opportunity to infect the glands and this study may have missed the

chance by leaving a two-day gap between day 15 and 17. Considering the variation in parasite development seen within the zygotes and kinetes and sporoblasts however, it is hard to believe that the window of opportunity for seeing kinetes penetrating the salivary glands could be so limited. Mehlhorn *et al* 1979 showed electron micrographs of *T. ovis* kinetes after having just penetrated salivary glands of *Rhipicephalus evertsi evertsi*. The images were very striking, and assuming the process with *T. parva* in *R. appendiculatus* is similar it would certainly have been possible to visualise it at the level of light microscopy. There were insufficient numbers of pathologically affected zygotes and kinetes to confirm specific killing mechanisms within the ticks. The rapid decrease of zygotes and kinetes after their numbers had peaked, without a concommitant increase in the number of kinetes and sporonts respectively, suggests they were somehow destroyed in the gut digestive cells. In their investigation on *Babesia* forms within *Boophilus microplus*, Potgieter *et al* (1976) described many immature vermicules in parasitophorous vacuoles in the gut epithelial cells. Some of the vacuoles were observed to contain fine granular material and traces of degenerating endoplasmic reticulum, apparently of parasitic origin. Other immature kinetes were seen in direct contact with the host cell cytoplasm as were the mature kinetes. *T. parva* forms were not observed within parasitophorous vacuoles in the course of this study, but there was a suggestion of *R. appendiculatus* defence mechanisms against the parasite. The amorphous material surrounding the kinete (Fig 5.13) was very similar to that described by Vernick *et al* (1995) in their study of refractory mechanisms in *Anopheles gambiae* to *Plasmodium gallinaceum*. Although the killing mechanisms were not elucidated, the ookinetes were killed in the cytoplasm of the midgut epithelial cells without melanotic encapsulation. *R. appendiculatus*-mediated destruction of *T. parva* zygotes or kinetes has not been previously described. Apart from the work carried out by Mehlhorn *et al* (1978), the zygote and kinete forms have not been studied in great detail *in vivo*. It is possible that some of the zygotes and kinetes Mehlhorn *et al* (1978) examined were actually suffering pathology, but this was not noted. Shaw *et al* (1991) conducted an electron microscopy study of *T. parva* sporozoite entry to, and survival in, bovine peripheral blood mononuclear cells. One aspect of particular interest was the parasite

mechanisms for escaping from the encapsulating host cell membrane formed during parasite entry. They described a 'dense fuzzy zone' on the outer membrane of the sporozoites as a result of rhoptry and microsphere discharge and that the discharge of the material was associated with the separation of parasite and host membranes. They stressed that the 'fuzzy material' was not composed of disintegrating host cell membrane. The amount of degenerated material seen around the periphery of the kinete shown in Fig. 5.13 is considerably greater than the parasite would be able to expel, suggesting the material is of host origin.

PCR results showed a large decrease in the detection of parasite DNA in the tick guts from day 0 to 5. Parasites were not seen in the sections during this time but the PCR indicates that this is likely to be the stage at which the greatest numbers of parasites are eliminated from the tick. This corresponds with the detection of parasite DNA in the faeces. An amplicon was detected at the lowest haematin granule mass (1×10^{-3}g) tested, showing that *T. parva* DNA must have been highly concentrated within the excreta. If the sexual stages have not crossed the digestive cell membrane by very early on in the engorgement period, the risk of being excreted is increased. Agbede *et al* (1984) showed *Babesia bovis* zygotes in the cytoplasm of gut epithelial cells of *Boophilus microplus* ticks on day 4 after engorgement. The variation in band size seen from day 5 onwards confirms the quantitative results from the histological sections, in that large variation appears to be a feature *of T. parva* infection in *R. appendiculatus* from the outset as opposed to just when it reaches the sporoblast stage. The band size increased slightly on day 12 and dropped by day 17. Again, this result seems to fit in with those from the sections. The zygotes will be increasing in size by day 12 (with a concomitant increase in the size of the nuclei and therefore volume of DNA) to a peak at around day 15, and then rapidly decreasing in number by day 17. The strong amplicon produced by one of the guts from a day 26 tick was unexpected. The tick had completed its post moult development by this stage and was an unfed adult. Although sporont contamination from the salivary glands cannot be categorically ruled out, it was considered unlikely as both the gut and salivary glands were dissected out whole. This may suggest that *T. parva* DNA remains in the

gut for considerably longer periods than is biologically functional. The amplicons seen in the salivary glands from day 16 onwards increased in intensity to day 26 (the final day of sampling). The band sizes from the salivary glands of day 26 ticks were larger than those from guts from day 0 ticks. The number of sporonts present in the adult salivary glands would have been many magnitudes less than the number of piroplasms in the guts of engorged nymphs. The stronger amplicons indirectly show the massive growth (and consequent increase in nucleic acid) which occurs at the sporont/sporoblast stage in unfed ticks.

The number of *Dermacentor variablis* haemocytes has been documented to increase in response to experimentally induced bacterial infections (Johns *et al* 1998). Although the figures showed a large range of haemocyte numbers, it was not as great as the range in numbers presented from this study (although the counts were made by completely different techniques). Since counts were not made from ticks before *T. parva* infection it is not possible to comment on their significance. It does indicate however, that it would be a worthwhile area for future investigation.

The structure and development of salivary glands were examined at various times throughout the moult, in histological section and in whole mount. This was valuable, as it is highly relevant to kinete penetration and sporont development. The rise in kinete numbers by day 15 (discounting the first day 8 peak because of complete tick mortality in that group) and the appearance of sporonts on Day 17 means the kinetes must have penetrated the glands around day 16. Because the acini are immature at this point, there are likely to display characteristics that make them suitable for *T. parva* penetration and development. Future work on aspects of salivary gland penetration by *T. parva* kinetes may benefit by examining the differences in *R. appendiculatus* salivary glands between days 15 to 19 (by which time kinetes appear unable to penetrate) post-detachment to establish the factors which permit parasite survival. Because the glands develop so rapidly in this period and the parasite invasion window of opportunity appears to be very limited, very regular sampling in this period may be necessary.

CHAPTER 6

Theileria parva induced pathology on *Rhipicephalus appendiculatus*

The aim of this chapter was to describe the pathogenic effects of *T. parva* infection on *R. appendiculatus*.

Introduction

There is less information available on the pathological effects of vertebrate parasitic diseases on their arthropod vectors than the subject deserves. Protozoan and helminth parasites cause disease in their vectors just as they do in their definitive hosts. Regarding vectors as nothing more than flying or crawling syringes is fast becoming an outdated view now that genetic manipulation of vectors and the enormous possibilities the concept allows is becoming more of a reality.

Pathology in ticks as a consequence of *Theileria* infection has not previously been documented in a highly detailed fashion. Schein and Friedhoff (1978) reported salivary gland pathology in *Hyalomma excavatum* ticks with *T. annulata* infections but the paper concentrated on aspects of *T. annulata* biology and the pathological effects on the tick were mentioned briefly in one paragraph. Büscher and Tangus (1986) reported that *R. appendiculatus* supporting very heavy *T. parva* infections are excluded from further propagation as they cannot feed due to the destruction or altered utilisation of their Type III acini. They suggested that all the acinar metabolic activity was being channelled into parasite propagation instead of the production of substances needed for feeding. This may be particularly true of the attachment cement which is predominantly produced from the e cells in Type III acini which are the preferred site for *T. parva*. These reports did not actually show images of the mechanisms causing the pathology. The aim of the work described in this chapter was to show that such damage occurs and that it reduces reproduction and survival of the ticks.

The results presented in this chapter clearly show the parasite induced pathological effects of *T. parva* on *R. appendiculatus* as they progress through the nymphal to adult moult. The histological sections used in this study were the same as those in the previous chapter but for the sake of clarity on the two subjects, they have been reported separately.

To determine at a microscopical level whether a cell is dead or dying requires examination of fine detail in subcellular structures. Information on the integrity of endoplasmic reticulum, mitochondria, nuclei etc. has to be ascertained before an informed decision can be made. Transmission electron microscopy can usually provide more information on these processes than light microscopy. Structures that are only vaguely suggested at the level of light microscopy can be properly defined with electron microscopy. The availability of appropriate control material is essential and was fulfilled by using uninfected ticks in this study. Previous light and electron microscopical work on *R. appendiculatus* gut tissue (Walker and Fletcher 1987), salivary glands (Fawcett *et al* 1981, Walker *et al* 1985) and *T. parva* forms within *R. appendiculatus* (Mehlhorn *et al* 1978) were used as guides when viewing the material.

Assessing parasite induced tick pathology is interesting in its own right, but the real significance of this aspect of the host-parasite relationship is to study the effect *T. parva* induced pathology has on tick behaviour, in terms of feeding performance and reproductive capacity. *Theileria* parasites are known to be very selective for the tick organs they penetrate and develop in, namely the gut and salivary glands. *Theileria* infection in ticks is not transovarial, thus the only instars capable of transmitting the disease are the nymphs and adults. However, the presence of the parasites within nymphs could conceivably have a detrimental effect on the reproductive capacity of the ticks, an effect that Chiera *et al* (1985) had previously demonstrated in association with host resistance. Reduction in fecundity has been reported for a number of arthropod species in relation to their parasite burdens.

Materials and methods

Calf and tick infection

The calf and tick infection data were as described in the previous chapter.

6.1. *Examination of sectioned material*

6.1.a. Light microscopy

The same methacrylate embedded tick samples prepared and examined in Chapter 5 were used for the purposes of this study. The infected tick sections were continually compared with those from the control, uninfected batch and examined under oil immersion at x1000 magnification.

6.1.b. Electron microscopy

A number of ticks were processed for transmission and scanning electron microscopy.

6.1.b.1. Transmission electron microscopy (TEM)

All the processing steps were carried out in a fume-chamber and the samples were kept rotating at all times.

After removal from Karnovsky's fixative, the samples were washed three times for 30 mins each wash in 0.2M phosphate buffer (see Appendix 6). They were then post-fixed for 60 mins in 1% osmium tetroxide diluted in 0.2M phosphate buffer. The samples were then stored in 0.2M phosphate buffer at 4^0C.

Three 20 min washes in distilled water were followed by dehydration through a graded acetone series of 50% - 10 mins, 70% - 10 mins, 90% - 10 mins, 100% - 3 x 10 mins. Epoxy resin (Araldyte type) was used for embedding. The embedding monomer mixture was diluted with dry acetone at 1:1 for 30 mins after which the samples were infiltrated with the mixture overnight at 60^0C. They were removed and placed directly in the embedding mix and further infiltrated for 4 x 60 mins. As per manufacturer's instructions, 0.5g of accelerator was added to the embedding mixture and the samples were infiltrated for 2 x 60 mins. The mixture was poured into

moulds, the samples were added and then kept at 60°C for 48hr to allow polymerisation to occur.

Gold coloured sections (approximately 60nm thick - the colour reflects the thickness of the section) were cut with a diamond knife using a Reichert OMU4 Ultracut ultramicrotome and mounted on copper grids (200 mesh). The sections were stained with uranyl acetate and lead citrate using an LKB Ultrostainer. The specimens were viewed using a Philips 400 transmission electron microscope.

6.1.b.2. Scanning electron microscopy (SEM)

After removal from Karnovsky's fixative, the specimens were washed in 0.2M phosphate buffer for 3 x 20 mins. They were then post-fixed for 120 mins in 1% osmium tetroxide diluted in 0.2M phosphate buffer. A thirty min wash in distilled water was followed by dehydration through a graded acetone series of 50% - 30 mins, 70% - 30 mins, 90% - 30 mins, 100% dry - 3 x 30 mins. The samples were critical point dried with CO_2 in a Poleron E300 SII Critical Point Drier. They were then sputter coated with 20nm gold/palladium (60/40) in an Emscope SC500 sputter coater. The specimens were viewed with a Philips 505 scanning electron microscope.

6.2. *Effect of* T. parva *infection on fecundity and hatch rate*

The reproductive capacity of adult ticks from the previous experiments was compared with those of control ticks.

The infected adults had moulted from nymphs that detached from calf 48A seven months previously. The piroplasm parasitaemias on the days of detachment were 3.2 - 13.1% and 13.1 - 19.6%. The control ticks were from the same original batch and had been fed on rabbits at the same time as the bovine feed of the infected ticks. The ticks were maintained at 18°C, 85% r.h until they were used. 60 male and 50 female infected and uninfected ticks were split into two batches of equal numbers. 30 males and 25 females were placed in ear bags on two NZW rabbits so that on one ear infected ticks were feeding and on the other ear, uninfected ticks. Because feeding

male ticks produce a pheromone that induces females to aggregate and attach (Rechav *et al* 1976, 1977), male ticks were placed in the bags one day before the females. The ears were examined two to three times daily, and engorged, detached females collected. The females were weighed and recorded, as was the bijoux tube and cotton wool bung in which they were kept. Females commenced egg laying at about day 5 and for the most part stopped by day 30. The bodies of the dead females were then removed and discarded. The tube was reweighed and egg batch weight calculated by subtraction. Larvae began to emerge from day 35 onwards and as they progressed in their post-moult development, migrated towards the tube bung that was generally away from the egg cases. A visual ranking of approximate hatch rate was made by examining the eggs with a stereo microscope.

Results

6.3. *Examination of sectioned material*

Days 0 to 8

No differences were noted between the infected and uninfected ticks in terms of their moulting performance or morphology as evidenced in the histological sections. Both groups of ticks followed developmental pathways as described by Walker and Fletcher (1987). The sessile digestive cells were massive and packed with very darkly staining protein vesicles. There was little convolution of the basal area of the sessile digestive cells and little accumulation of residual bodies within them. The lumen of the gut remained occluded with the distended sessile digestive cells and the motile digestive cells.

Days 9 / 10

By day 9 a vacuolated appearance of the basal area of the sessile digestive cells was seen in the histological sections of infected ticks. This seemed to be caused by a coalescence of lipid vesicles into large irregular shapes.

Examining the day 9 infected ticks revealed that their moult appeared to be slowing down compared to that of the controls. This was evident by their overall darker

colour, suggesting delayed bloodmeal digestion over the controls and less moulting fluid was visible around their anterior end which was becoming translucent in the controls.

Day 12

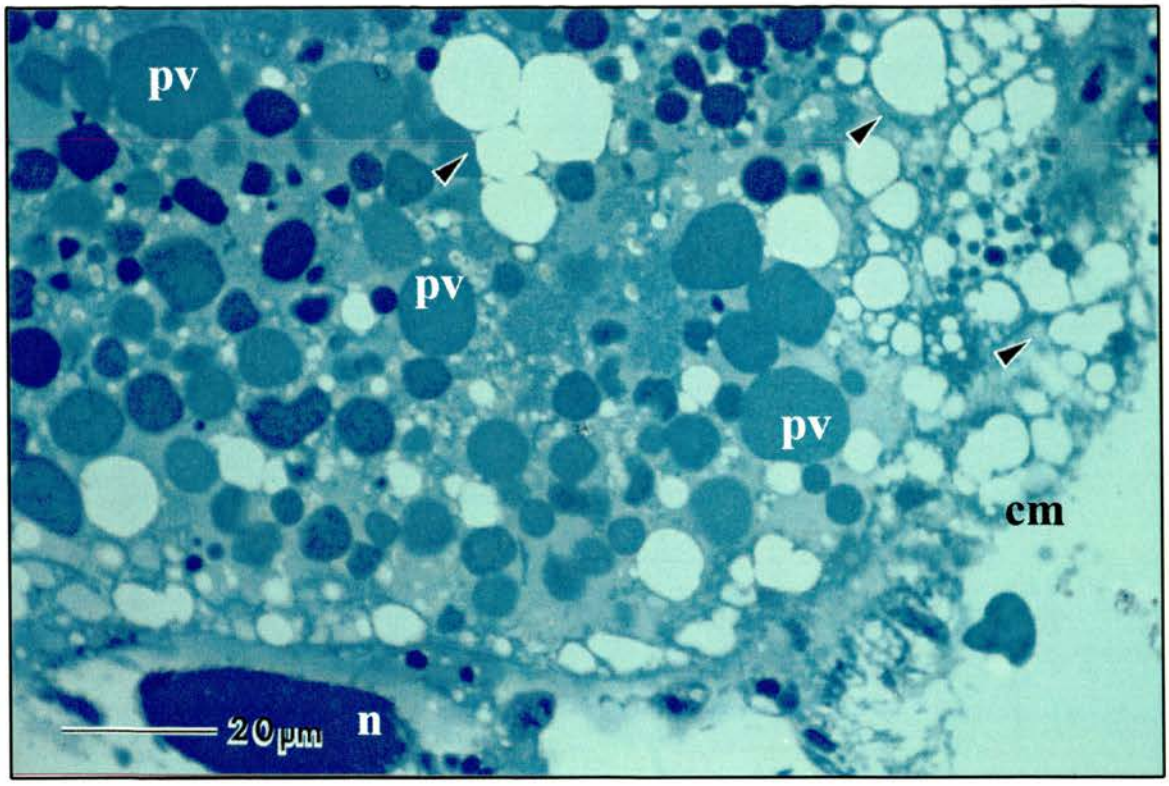
Sections showed that the digestive process in the infected ticks was behind that of the controls. The homogenous gut contents of the controls contained a greatly increased amount of small and large residual bodies. This was in contrast to the infected ticks, where the gut contents were very heterogeneous and showed highly compartmentalised digestive processes which was not apparent in the controls. Large residual bodies were present but small residual bodies were completely absent. Fig. 6.1a shows a vacuolated section of gut from an infected tick. A comparable gut section from a day 12 control tick is shown in Fig. 6.1b. The protein vesicles in the control tick are considerably smaller than those in the infected tick and lipid is more evenly distributed throughout the gut. Green residual bodies can also be seen accumulating in the control tick gut which are completely absent from that of the control.

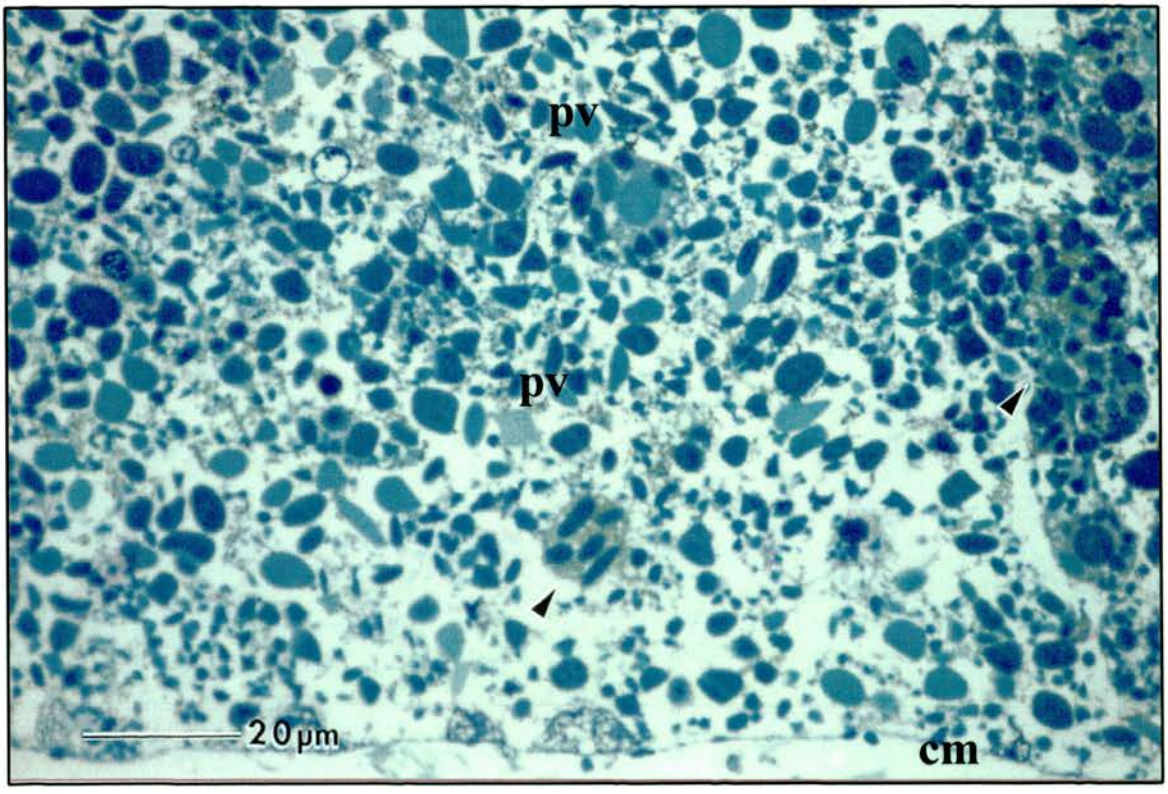
Examining the moulting day 12 infected ticks showed that their moult appeared to have completely halted and the ticks seemed moribund. They were darker in colour than on day 9, suggesting compaction of the bloodmeal, but no moulting fluid was seen in any of the ticks.

All of the infected ticks up to this point had been used from a single batch that detached at a piroplasm parasitaemia of 19.6 - 22.4%. The detachment numbers on this particular day had been so high that it should have been possible to use a continuous batch throughout the entire infected tick series. Because their moulting performance was becoming so much behind that of the controls however, this batch was abandoned as a source of sample material because it would be less comparable with normal moulting ticks. It was also noted that the mortality in this batch was around 33% on day 10 and rising daily. This level of mortality had never been seen

Figure 6.1a. Lipid vesicle coalescence in a gut digestive cell in a day 14 post-detachment nymph-adult. The gut is highly vacuolated with large irregularly shaped lipid vesicles (arrowheads). p v – protein vesicles, n – nucleus, cm – cell membrane.

Figure 6.1b. Gut section from a control, uninfected, day 14 post-detachment adult. Protein vesicles (pv) within the gut digestive cell are considerably smaller because of the much progressed digestion and large, green residual bodies are beginning to accumulate (arrowheads). Lipid vesicles were seen in these guts, but did not form the large irregular structures demonstrated in Fig. 6.1a. cm – cell membrane.


pv
pv
pv
cm
20μm
n


pv
pv
20μm
cm

previously with any other *R. appendiculatus* batch at the CTVM. A batch of larvae that detached on the same day had shown 100% mortality by this stage.

Ticks that detached on other days from the same calf were now used as sample material because on the whole they appeared to be exhibiting more normal moulting behaviour.

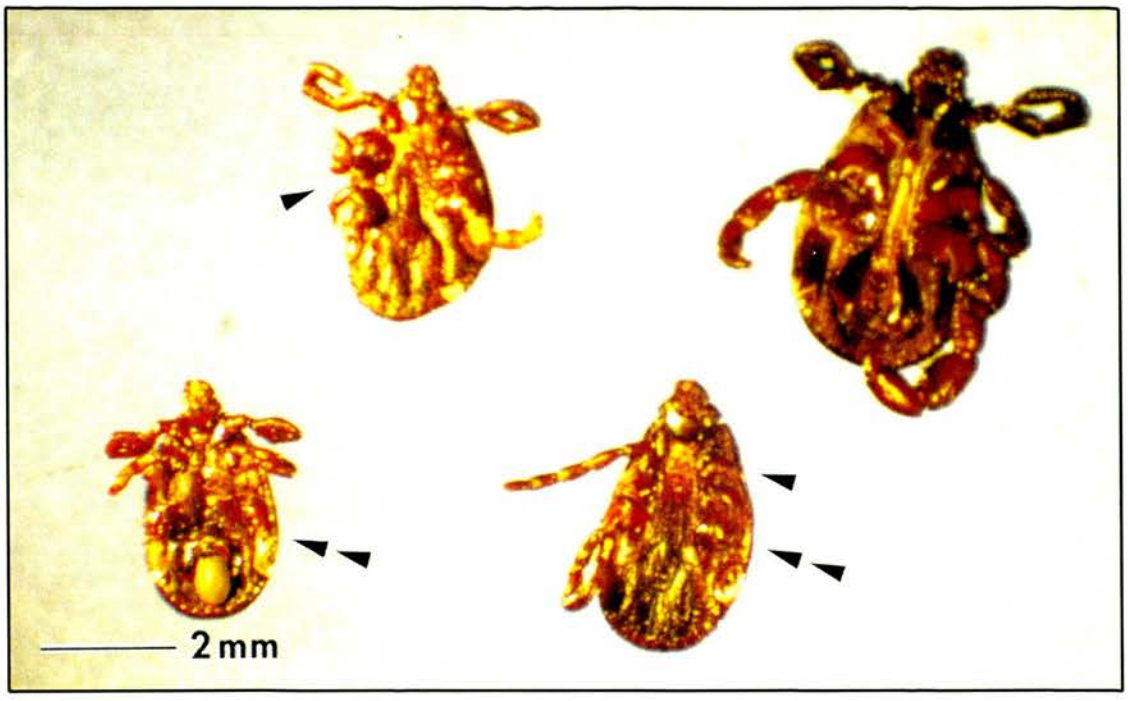
Days 13 / 14

Some of the ticks had emerged or were in the process of doing so by day 13. As has been reported previously, the females were seen to emerge a day before the males. Many abnormalities were noted in the infected ticks during the dissections on this day. Around half of the non-emerged, infected ticks (mostly males) had abnormal legs (Fig. 6.2). Yellow plaques were seen around the internal openings of the coxae on the nymphal exoskeleton and appeared to be preventing the normal development of the developing adult limbs.

Once the gut was removed, in a proportion of the infected ticks, a number of very large, brown coloured cells were seen very evenly distributed throughout the remaining tissue of the haemocoel. They were very much larger than haemocytes (as well as being the wrong colour) and looked like motile, gut digestive cells. The cells had not been released from the gut as a result of the dissection process, as the gut tissue in many cases was strong enough to be removed without tearing and because any cells released as a result of dissection would be very localised within the haemocoel.

The salivary glands of the infected ticks showed retarded development compared to the controls. There was a large variation between their development, but many of the glands looked no more developed than those in day 8 or 10 control ticks.

Figure 6.2. *Theileria parva* induced deformities in adult ticks. Twisted and stunted legs (arrow) and missing legs (double arrow) were commonly seen in affected ticks.



The mortality within this group of moulting nymphs was also becoming very pronounced. A batch of larvae that detached on the same day as the nymphs processed here showed 100% mortality.

The sections showed that the infected ticks were progressing with the digestive process. Protein vesicles were beginning to become slightly smaller and less numerous

and there were more lipid vesicles present than before. Large, abnormal vesicle structures were noted around the periphery of the sessile digestive cells. The infected ticks contained more protein vesicles, fewer lipid vesicles and fewer residual bodies than the uninfected ticks (see Walker and Fletcher 1987), although small residual bodies were starting to appear in the sessile digestive cells. Motile digestive cells were more prominent than before in control ticks and contained large amounts of residual bodies. The lumens of infected ticks were massively congested with protein vesicles without showing the developing structures of the motile digestive cells seen in the uninfected ticks.

Day 15

The sections revealed that the salivary glands of the controls look almost fully formed which is in marked contrast to those of the infected ticks.

The heterogeneous and protein dense nature of the infected ticks' digestion was extremely prominent. The contrast with the guts of control ticks was very marked (Figs. 6.3a and 6.3b). The protein vesicles in the control ticks' guts were becoming substantially smaller and the lipid content was increasing noticeably as were the amount of vesicle-bound large residual bodies. This gave the gut an appearance of being much less packed and advanced in the digestive process.

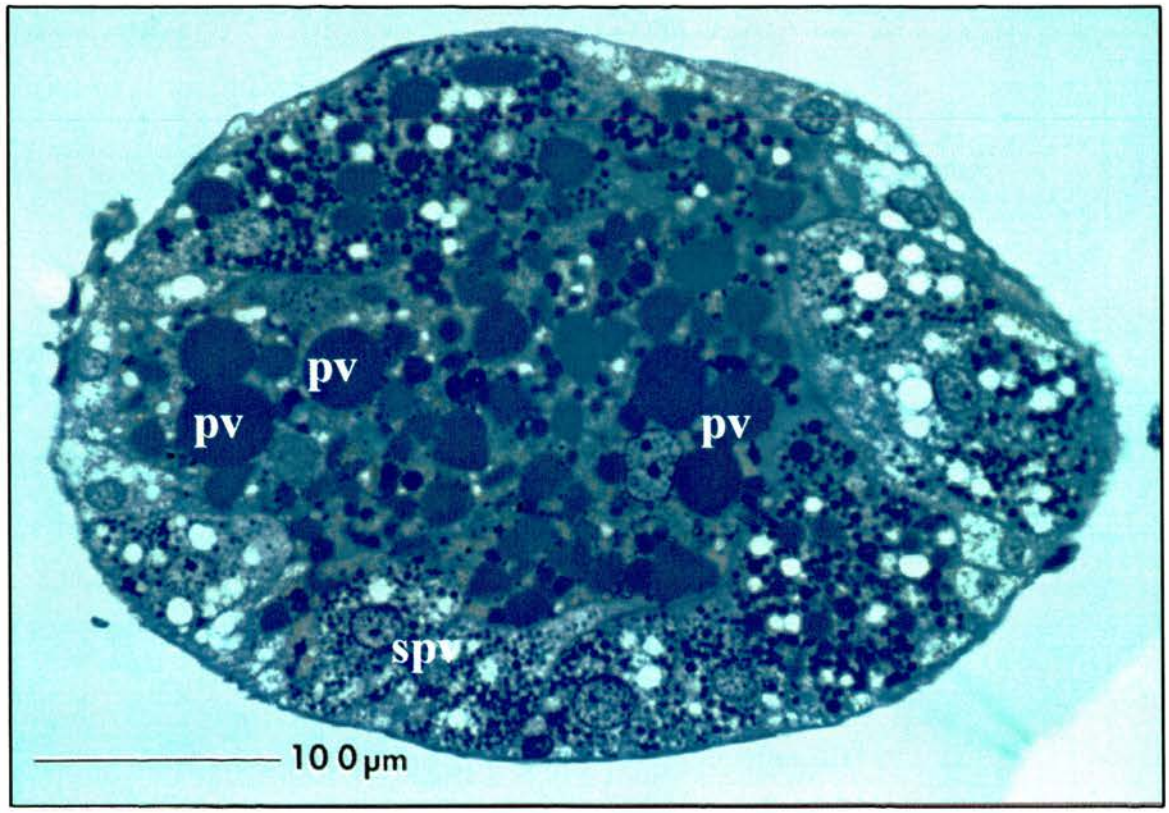
Motile digestive cells, very densely packed with large, residual bodies were seen free in the haemolymph (Fig. 6.4). These cells were gut derived and present in the haemocoel without any obvious signs of gut wall damage as a consequence of dissection. Although these cells were seen in histological section, and the others in the body of dissected ticks seen with a stereo microscope, the size and appearance of the cells were very similar to those seen dispersed throughout the haemocoel in a number of infected ticks.

Malphighian tubules filled with protein vesicles and digestive products were seen in a number of sections (Fig. 6.5). There also appeared to be structural deformities in some of the tubules (Fig. 6.6). Greatly thickened vessel walls reduced the lumen sizes and malformed tissue appeared to be obscuring the remaining area. Some of the ticks were quite obviously becoming constipated with large accumulations of guanine in the rectal sac. Whether this was caused by tubule congestion and blockage as a result of the above mentioned pathologies is not known, but is a possibility. None of these effects were seen in any of the control ticks.

Salivary gland acinar pathology was first seen in day 15 infected ticks. This was manifest mainly in the presence of metachromasic and pycnotic bodies (abnormal

Figure 6.3a. Gut section from a *T. parva* infected, day 15 post-detachment adult. The size of protein vesicles varies greatly in the gut section with very small, densely basophilic vesicles (spv) present around the periphery and much larger, less digested vesicles (pv) in the centre of the gut.

Figure 6.3b. Gut section from an uninfected, day 15 post-detachment adult. The digestive contents are more homogenous than in the gut from the infected tick (shown above). The accumulation of large, green residual bodies (rb) contrasted with their highly dispersed distribution in the infected tick gut. pv – protein vesicles.


pv
pv
pv
spv
100 µm

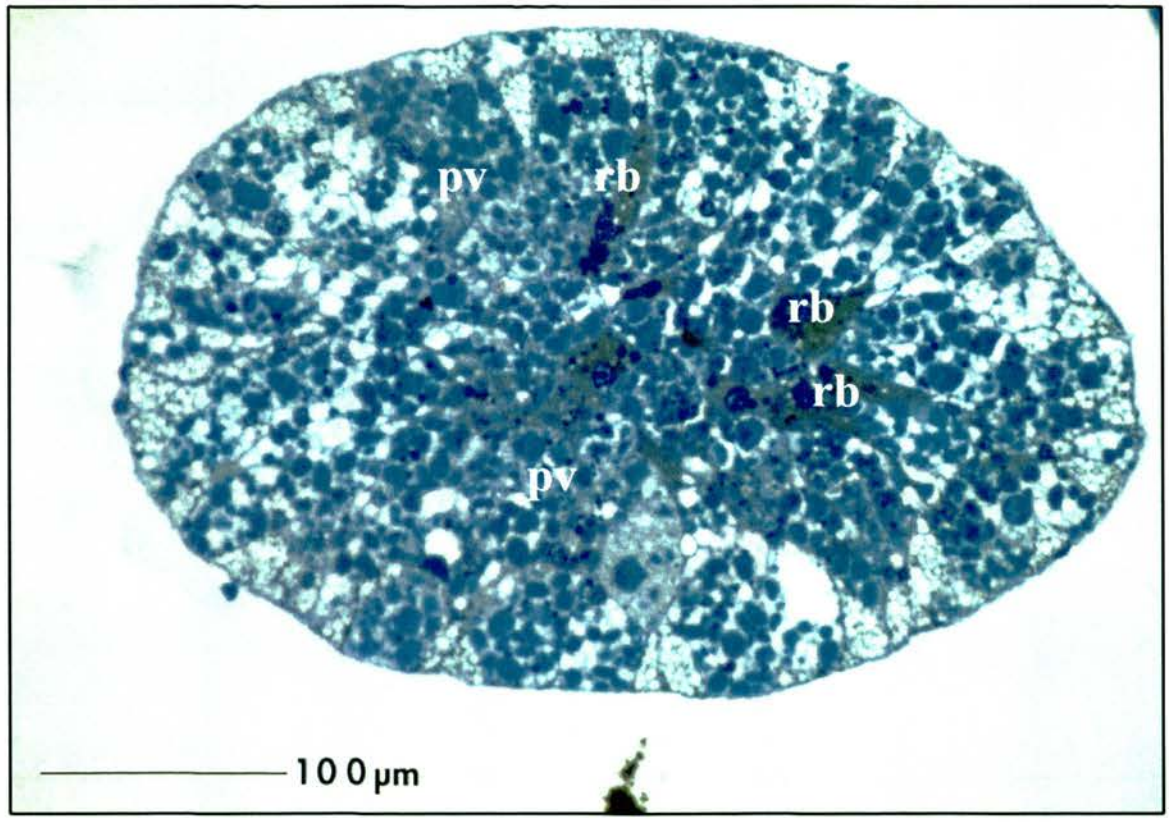
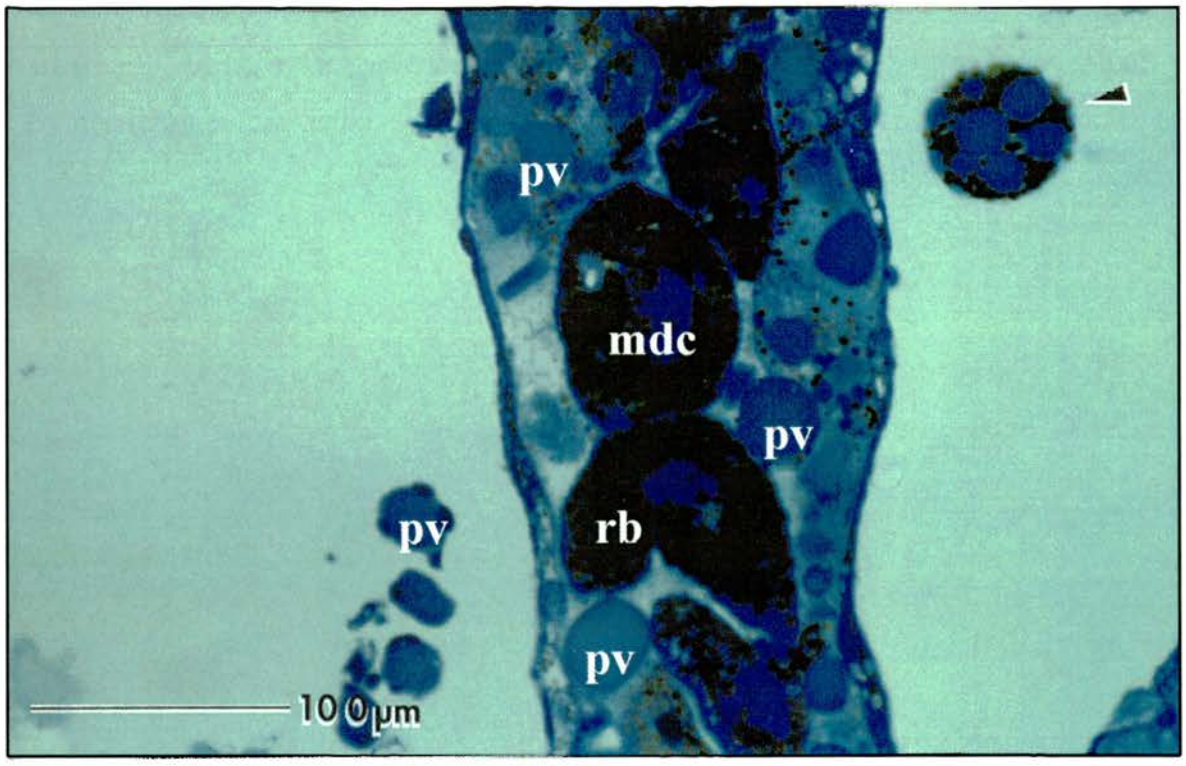

pv
rb
rb
rb
pv
100 µm

Figure 6.4. Gut section from a *Theileria parva* infected, day 16 post-detachment adult. Motile digestive cells (mdc) are very densely packed with small residual bodies (rb). The arrowhead indicates a gut derived, motile digestive cell free in the haemolymph. pv – protein vesicles.

Figure 6.5. Malphighian tubule in a day 15 post-detachment adult displaying very distinct pathology. Large protein vesicles (pv) and a motile digestive cell can be seen within the lumen (arrow).


pv
mdc
pv
pv
rb
pv
100µm

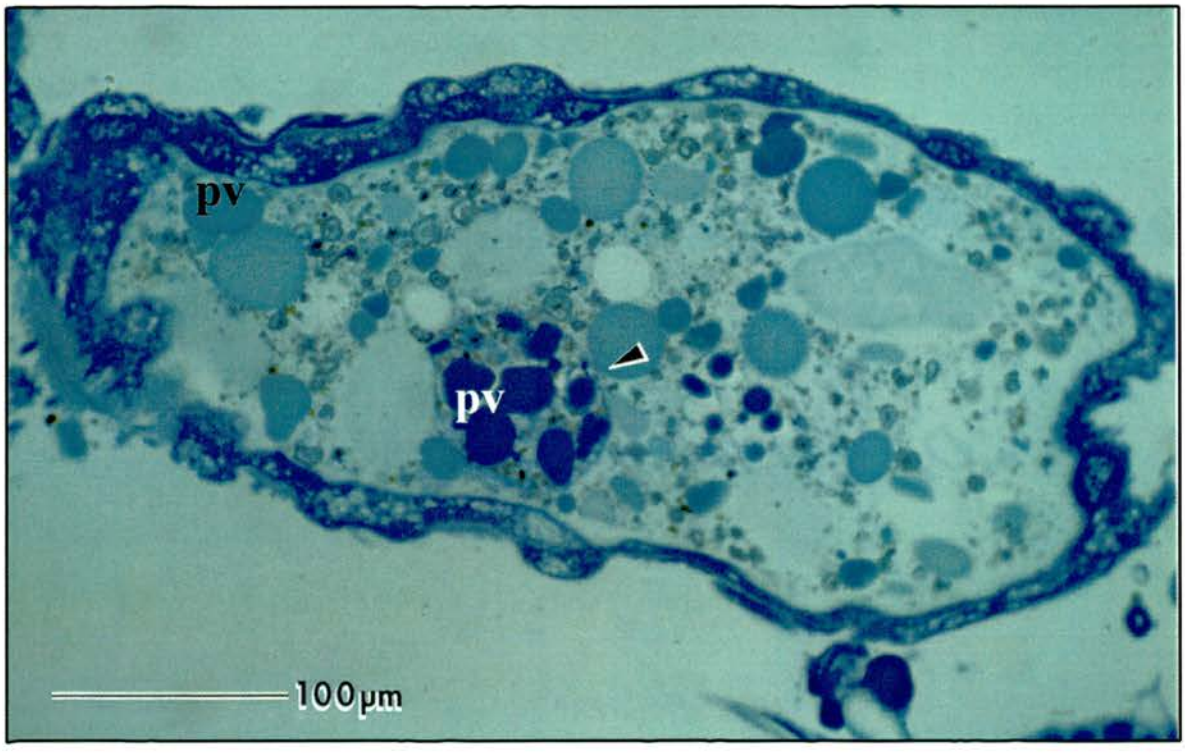
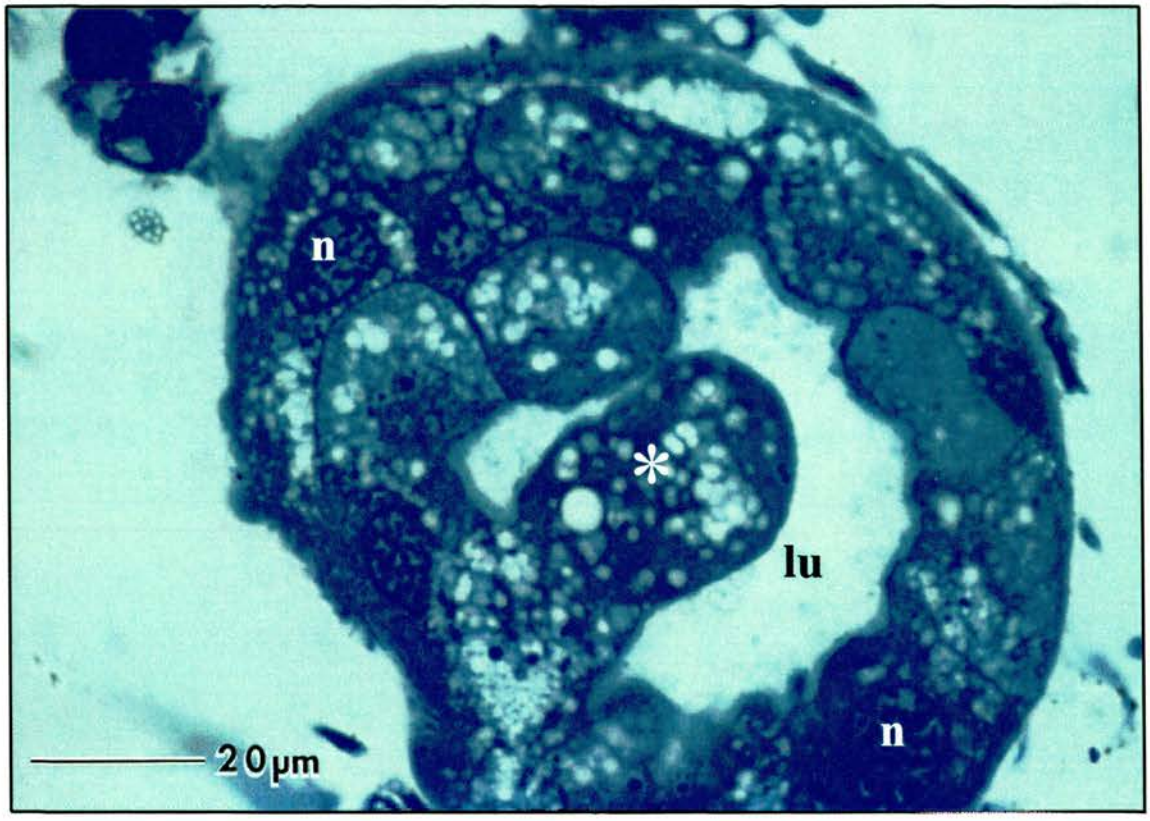
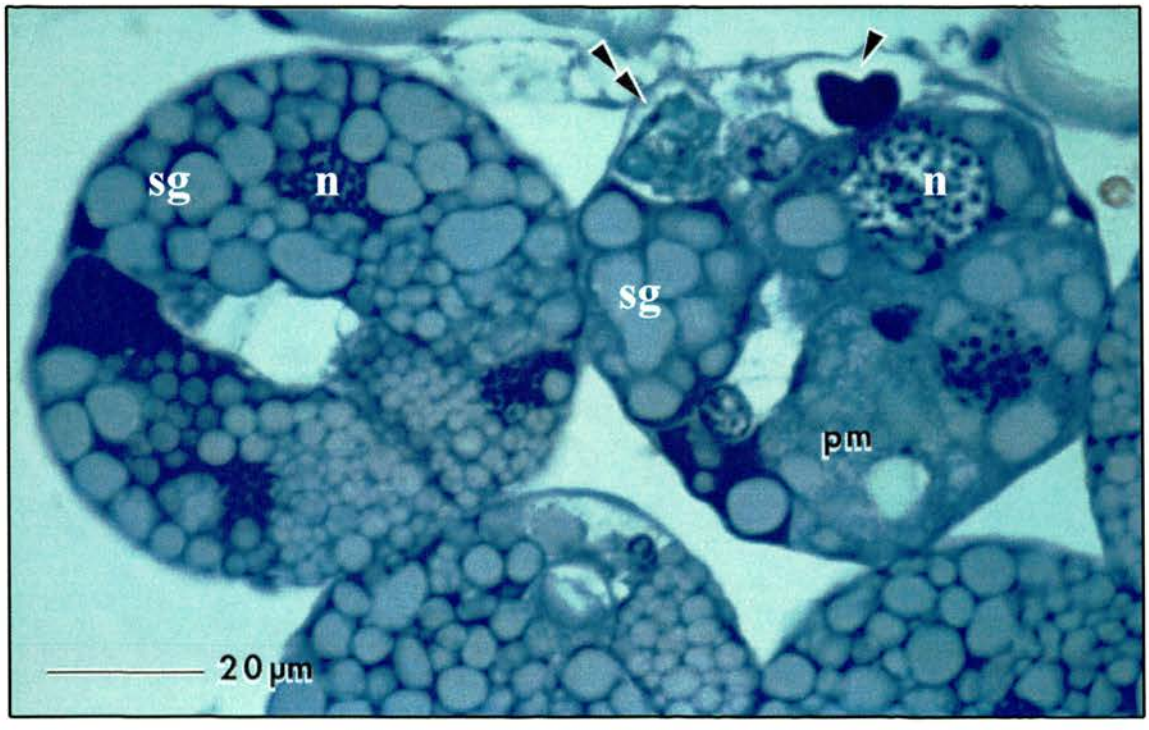

pv
pv
100µm

Figure 6.6. Malphighian tubule in a day 15 post-detachment adult showing a tumour-like process protruding into the vessel lumen. The vessel walls are greatly thickened compared to those of control ticks. lu – lumen, * - indicated the tumour-like obstruction, n – cell nucleus.

Figure 6.7. Pathologically affected Type III salivary gland acinus in a day 17 post-detachment adult. A metachromasic body (double arrowhead) within a dead cell, and a pycnotic body (arrowhead) in an adjacent dead cell are shown. Both objects suggest they were the acinar cell contents that have retracted from the outer membrane, possibly as a result of parasite invasion. The bottom e-cell looks like it contained a developing sporoblast (pm) indicating the acinus may have been subject to multiple kinete penetration. sg – secretory granules, n – nucleus.


n
*
lu
n
20µm


sg
n
n
sg
pm
20µm

staining colour, often green and dense) within the acini (Fig. 6.7). Because the acini were still immature by this stage, identification of affected acinar types and cells was difficult, but in the main, e cells in Type III acini appeared to be worst affected. The bodies within the acini appeared to be dead or dying host cells that were withdrawing from the outer membrane. Large, secretory granules typical of those found in e-cells can be seen in a number of affected acini.

Day 16

These ticks belonged to the batch that had been discontinued from regular sampling on day 12 because of early pathological signs. They were sampled again because they were so obviously moribund, thus it was best to use them promptly. The mortality within this group approached 80% by this stage. Every tick in this batch displayed a massive build up of guanine in the rectal sac that they appeared unable to excrete (Fig. 6.8).

The sections showed the guts were highly vacuolated and packed with very small basophilic protein vesicles that were not seen in control ticks at any point in their moult (Fig. 6.9).

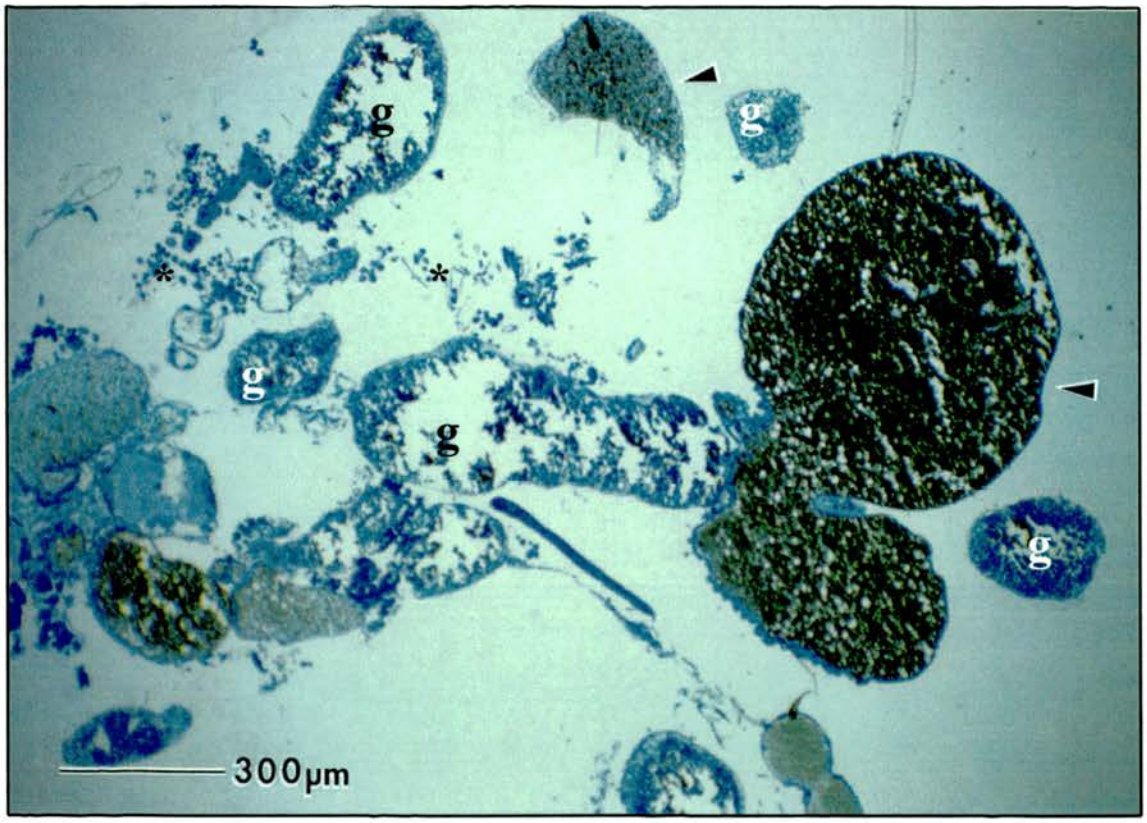
Days 17 to 22

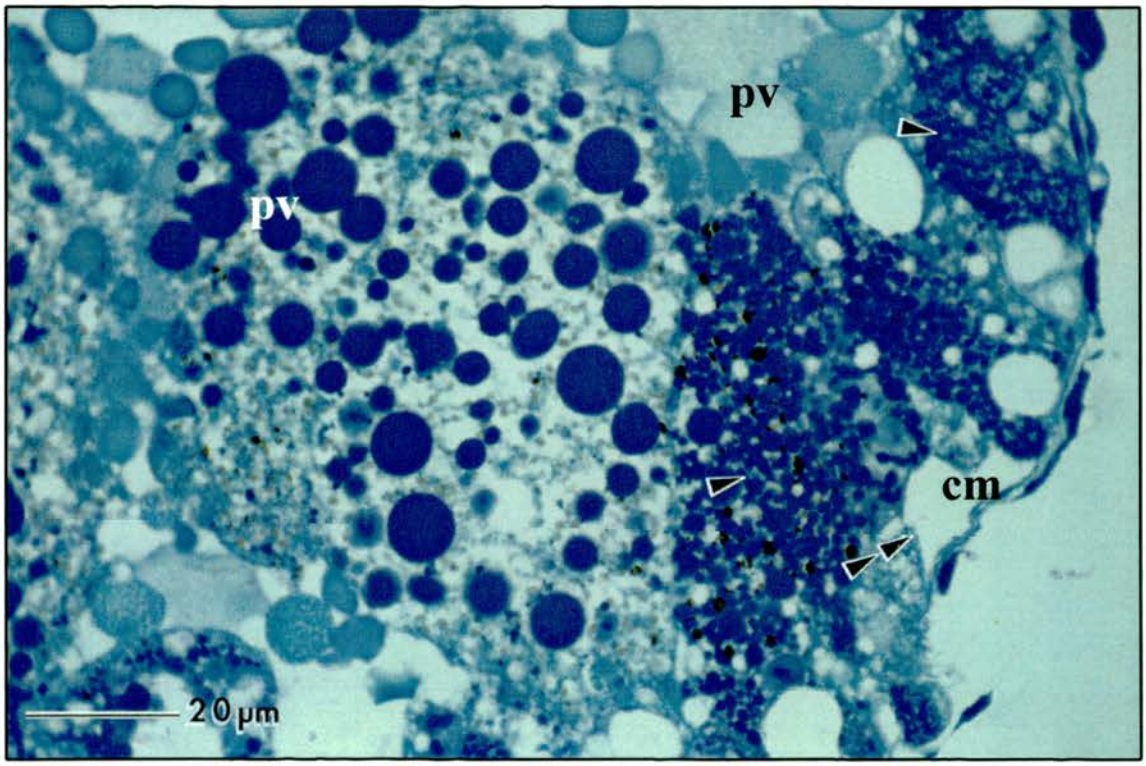
The uninfected ticks showed considerable changes in the sessile digestive cells with reduction of protein vesicle size and number and with massive accumulation of lipid vesicles. The basal area of these cells had become highly vacuolated and many small residual bodies had accumulated. The number of motile digestive cells decreased markedly leaving the gut lumens large, open and cell free.

Infected ticks showed a large variation in their rate of digestion with some guts resembling those of day 14 control ticks while others appeared more in line with those of the uninfected ticks at the same point in their moult.

Figure 6.8. A section through a morbid, constipated day 16 post-detachment moulting nymph. The rectal sac (arrow) is so large it dwarfs the gut and other tick organs. The salivary glands (*) were underdeveloped compared to that of control ticks. g – gut caecae.

Figure 6.9. Gut section from a *T. parva* infected, day 15 post-detachment adult. An accumulation of small, densely basophilic protein vesicles (arrow) and extensive vacuolation (double arrow) can be seen. The small protein vesicles were not seen in any control ticks throughout their moult. pv – protein vesicles, cm – cell membrane.


g
g
g
g
g
*
*
300µm


pv
pv
cm
20µm

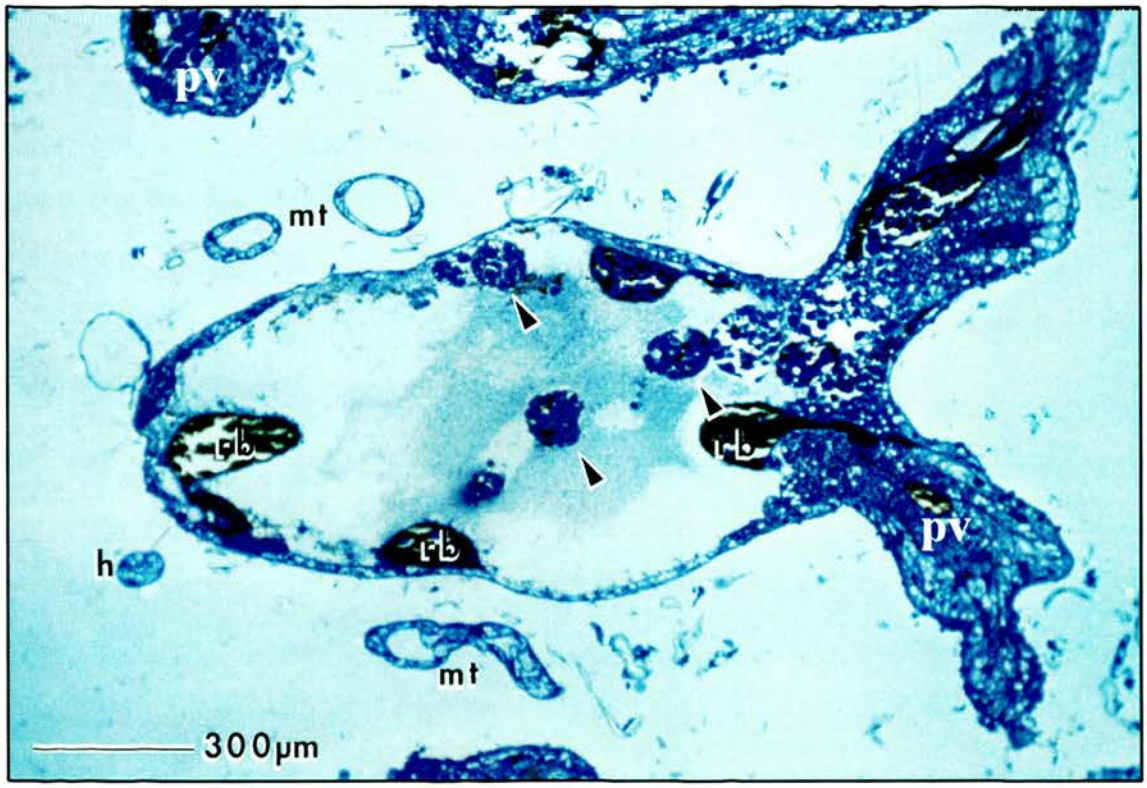
In general there were fewer changes in the sessile digestive cells and a greater accumulation of the small, basophilic protein vesicles noted in day 16 infected ticks.

There were fewer protein and more lipid vesicles than previously but there was very little accumulation of small residual bodies. The lumenal areas were less congested than previously, but they displayed fewer open, cell free spaces than the uninfected ticks. On dissection of infected ticks, it was noted that the guts were very fragile in comparison with the controls that commonly resulted in tearing. The sessile digestive cells appeared to lack well-defined structure that made it difficult if not impossible to delimit one cell from another because the membranes were not apparent. In some sections, it appeared that the basal lamina was the only structure preserving the integrity of the epithelium (Figs. 6.10 and 6.11).

Salivary gland pathology had become more advanced in the day 17 infected ticks. As well as dead or dying cells, large vacuoles were seen in Type III acini with a translucent appearance (Fig. 6.12). A number of acini were seen which had a completely degenerated structure and would have been functionally useless. Some of these cells may have contained primordial sporoblasts, but the compacted and pathological appearance of the cells made certain identification impossible. On dissection, the salivary glands of infected ticks were often seen to be less well developed than those of controls, with very few acini being present along the branched ducts, and often only at the distal ends. The acini were reduced in number and diameter compared to control ticks. Pathological effects were entirely confined to Type III acini as demonstrated in Fig. 6.13. Transmission electron micrograph images of Type III acini revealed that the affected cells retracted from the outer acinar membrane and became compacted and very dense (Figs. 6.14a and 6.14b). These images showed the cells juxtaposed to the affected cells (which were unidentifiable because of the pathology) appear healthy and functional. However, in some cases entire acini are destroyed (Fig. 6.15). The significance of these pathologies can be fully appreciated when affected whole mount salivary glands are viewed in context with healthy glands (Figs. 6.16a and 6.16b).

Figure 6.10. Highly degenerated gut structure of a *T. parva* infected, day 16 post-detachment adult. Little or no cellular structure can be seen. A few motile digestive cells (arrows) and dense collections of residual bodies (rb) are present, but the central lumen is almost completely empty. mt - Malphighian tubules, h – haemocyte, pv – protein vesicles.

Figure 6.11. Highly degenerated gut wall structure in the caecum from a *T. parva* infected day 16 post-detachment adult. Much of the peripheral structure appears to be maintained only by the basal lamina. A number of protein vesicles appear to be outwith digestive cells (arrows). pv – protein vesicles, lu – gut lumen.


pv
mt
rb
rb
rb
pv
h
mt
300µm

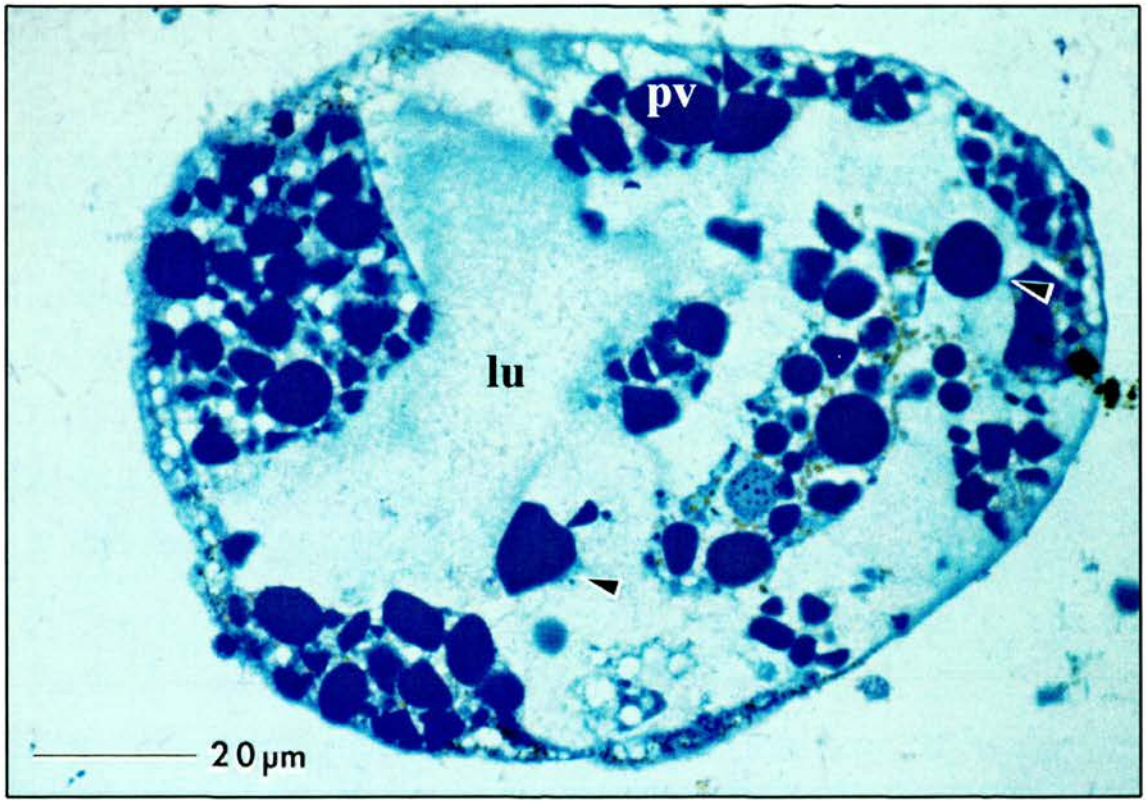
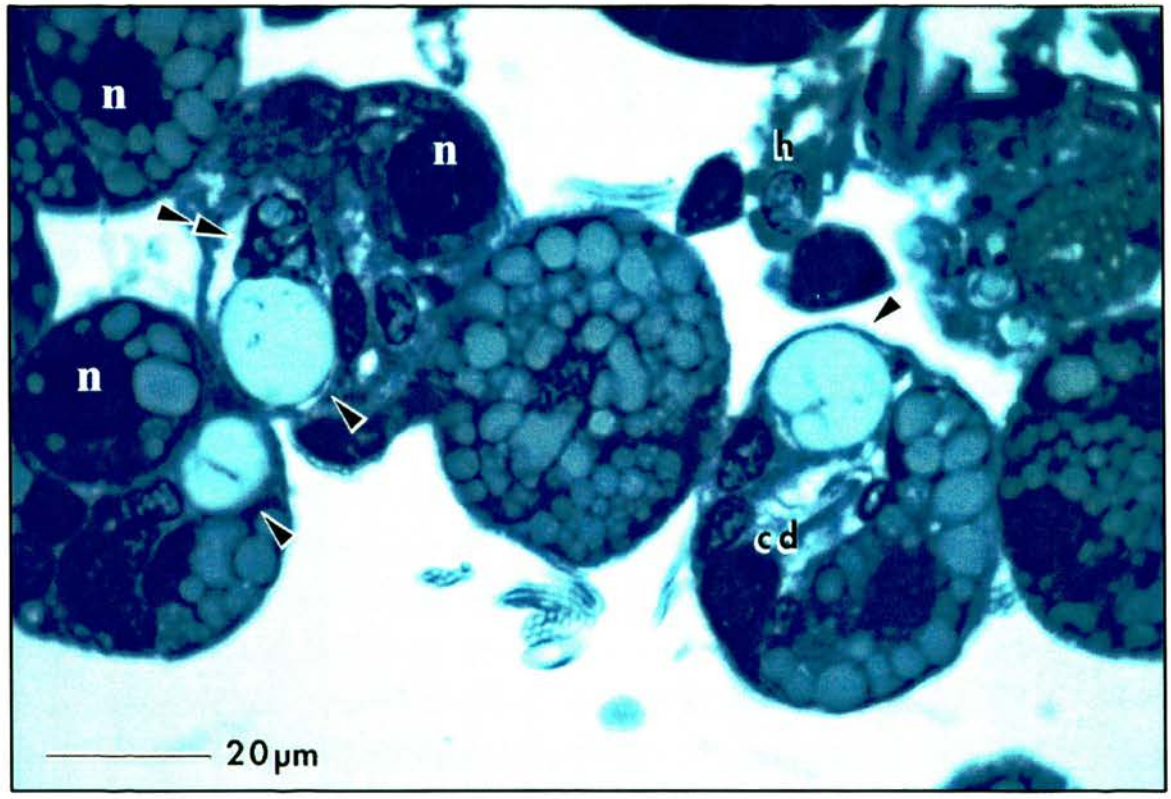

pv
lu
20µm

Figure 6.12. Type III acini in the salivary glands of a day 19 post-detachment adult displaying pathological effects. The arrows point to clear, translucent vacuoles and the double arrowhead shows a metachromasic body. The acinus on the right hand side of the photograph shows the vacuoles are clearly distinct from the acinar central duct (cd) and may represent cellular destruction as a result of parasite penetration. h-haemocyte within the surrounding haemolymph, n – acinar cell nucleus.

Figure 6.13. Type II (II) and Type III (III) acini in a day 19 post-detachment adult demonstrating the pathological effect found exclusively in Type III acini. The arrow points to a clear, translucent vacuole.


n
n
n
lh
cd
20µm

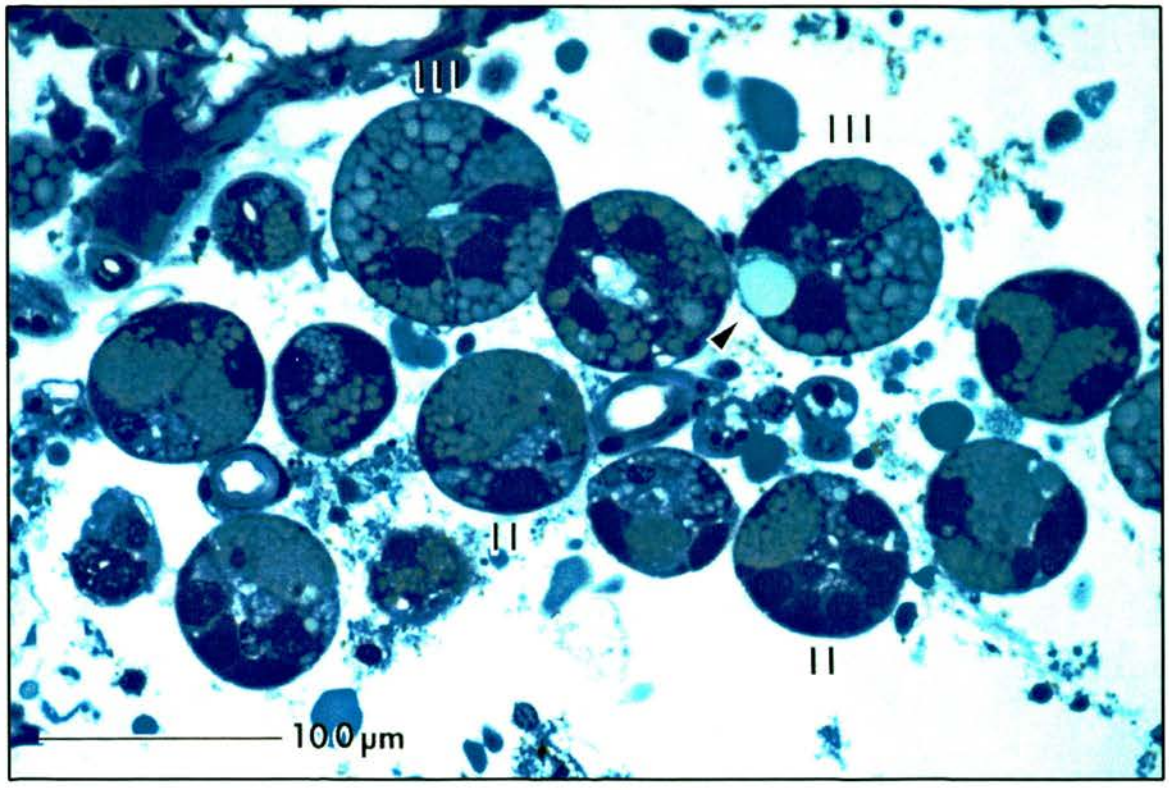
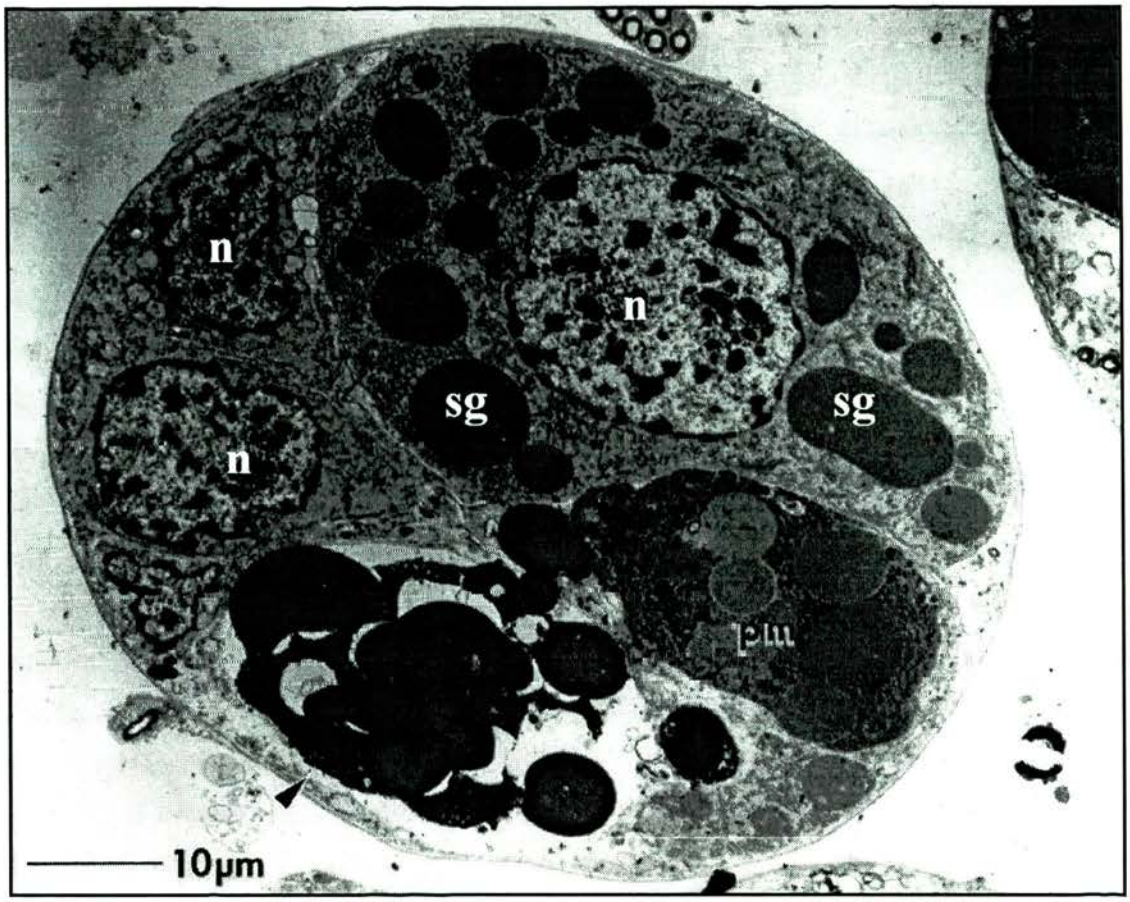

III
III
II
II
100µm

Figure 6.14a. Transmission electron micrograph of a pathologically affected Type III acinus from a day 19 post-detachment adult. The arrow points to a highly compacted, dead cell which had contracted from the acinar membrane. A suspected parasite mass (sporont) is present within the adjacent cell (pm). The sporont is compacted and shows a degenerating structure indicating it may have been the target of a tick mediated defence mechanism. n – acinar cell nuclei, sg – secretory granules.

Figure 6.14b. High power electron micrograph showing the contrast in cellular organelles between a healthy and dying salivary gland acinar cell, possibly as a consequence of *T. parva* infection. Well-defined endoplasmic reticulum (er) can be seen in the healthy acinar cell. The cell to the right is contracting from the cell membrane (cm) and exhibits dense, amorphous endoplasmic reticulum (der) clearly indicating cell death. sg - secretory granules in e-cells of Type III acini.


n
n
n
sg
sg
10μm

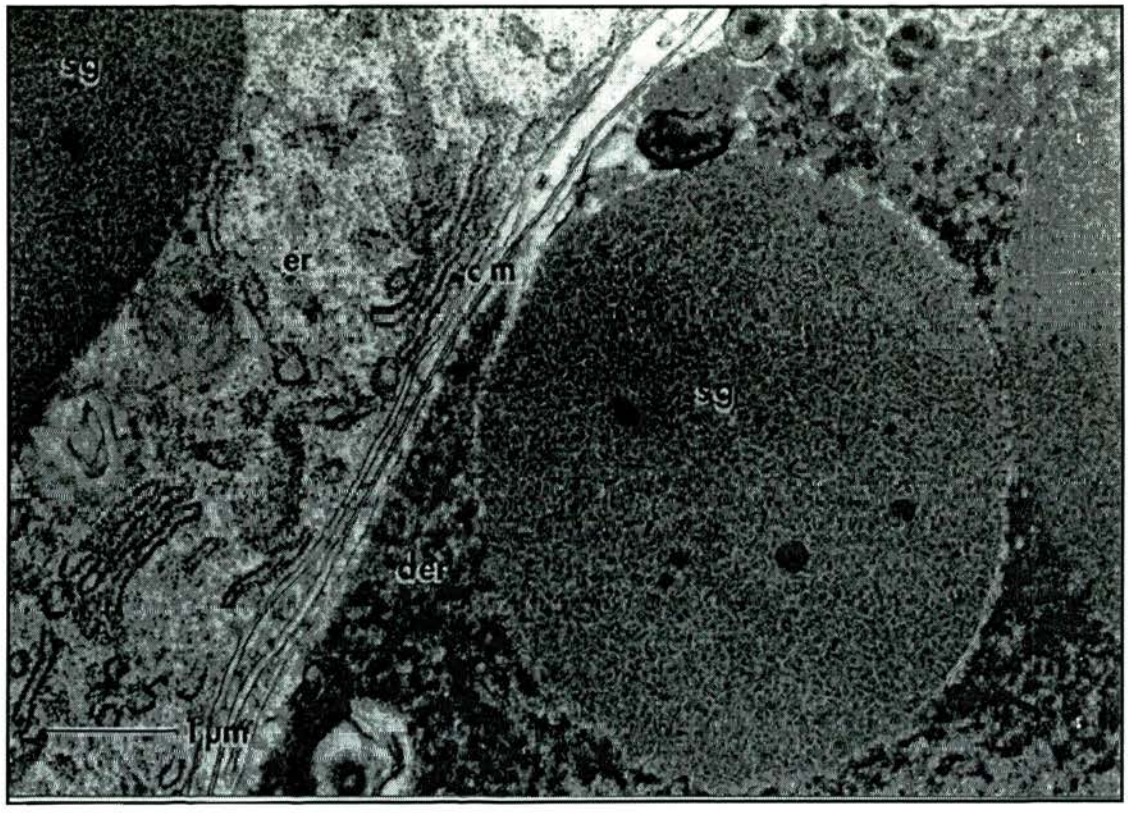
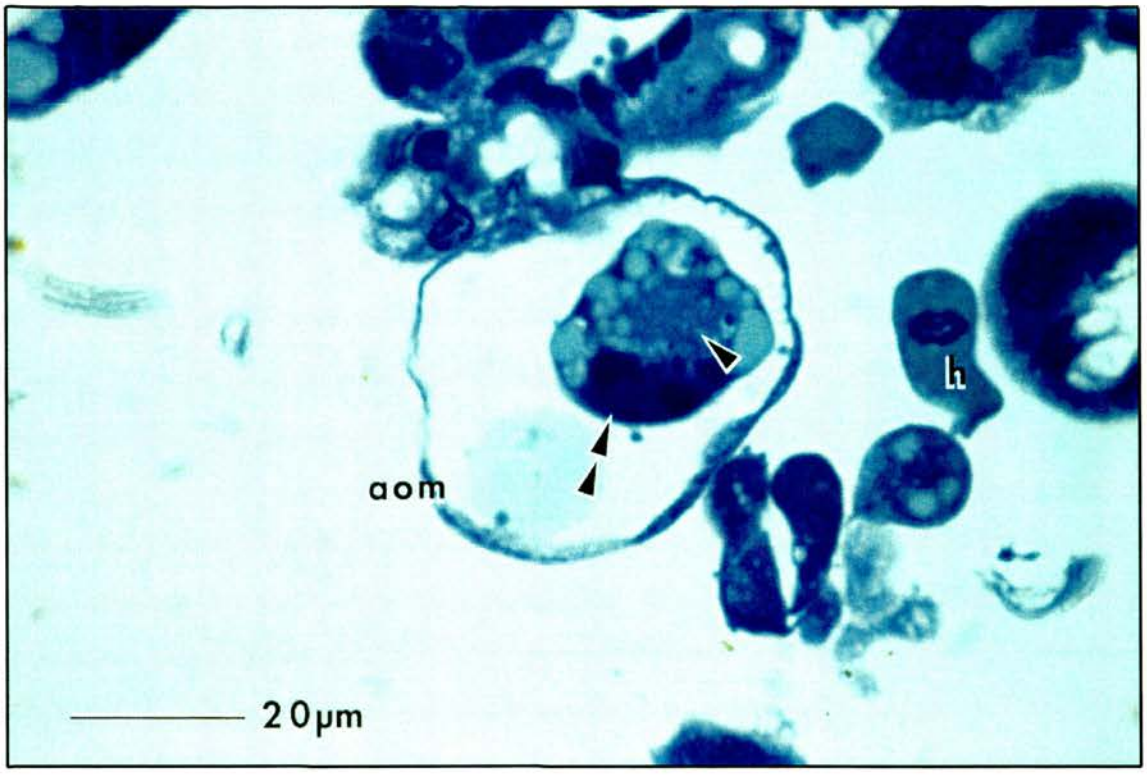

sg
er
cm
sg
der
1μm

Figure 6.15. The contents of a dying salivary gland acinus from a day 19 post-detachment adult retracted from the outer membrane. Figs. 6.12 to 6.14 showed pathology in single cells within affected acini. However, some acini were affected to the extent that the whole acinus died. The photograph opposite shows an example of this. The arrow points to a suspected sporoblast in the central cellular mass, which is likely to have caused the pathology. Dense, basophilic bodies (double arrow) indicate cell death and compaction. h – haemocyte, aom – acinar outer membrane.



The pathologically affected glands have bare ducts and the few acini that are present tend to be distally located. Day 17 saw the first appearance of sporonts in Type III acini. No evidence of kinete invasion was seen before this.

Haemolymph

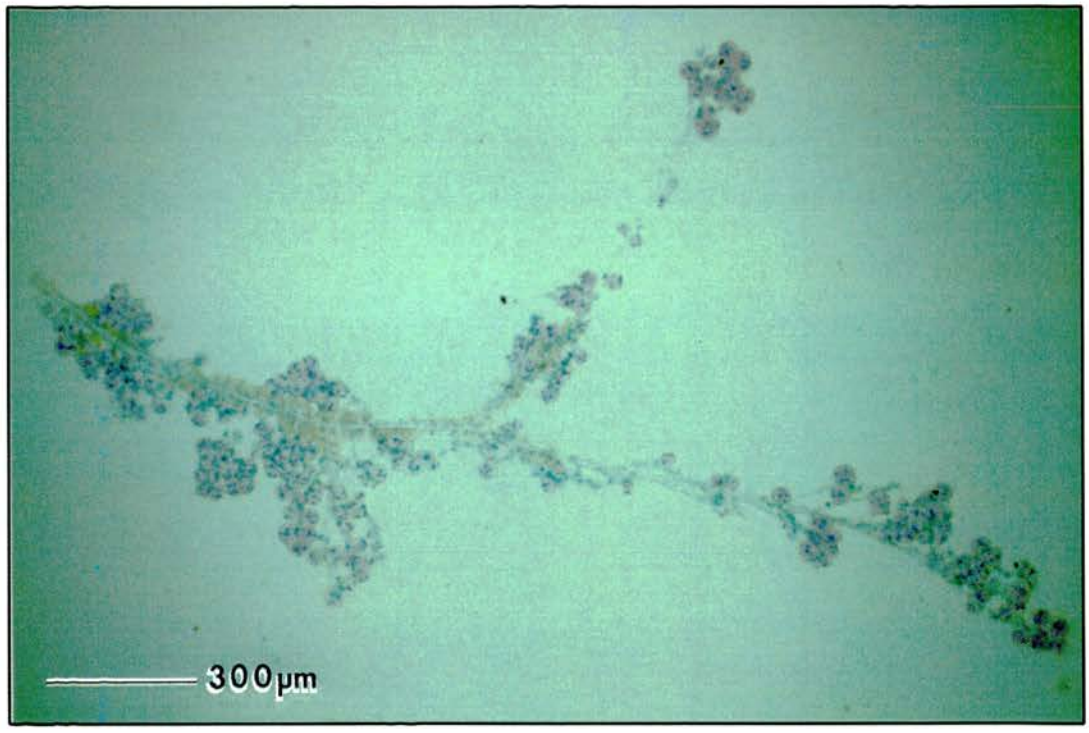
The numbers of haemocytes in the sections were not counted. The apparent density of the haemolymph and numbers of haemocytes with basophilic and eosinophilic granules appeared similar in both uninfected and infected ticks at all developmental stages.

Malphighian tubules

Malphighian tubules in infected ticks did not generally display the obvious pathology seen in the gut and salivary glands. On the whole, their development closely paralleled that of the control ticks. Other than the pathology documented earlier (day

Figure 6.16a. A pathologically affected salivary gland showing very few acini from an adult that had completed post-moult development. Long stretches of the ducts are bare of acini, and the few acini that are present tend to be distally located. No sporoblasts were detected in the gland.

6.16b. A healthy salivary gland from an adult that had completed post-moult development. The arrows indicate just 4 sporoblasts in a gland that is heavily infected with *T. parva*. Comparison between the two glands reveals an enigma in terms of pathology; why some glands support high infections without any obvious deleterious effects and others do not?


300µm

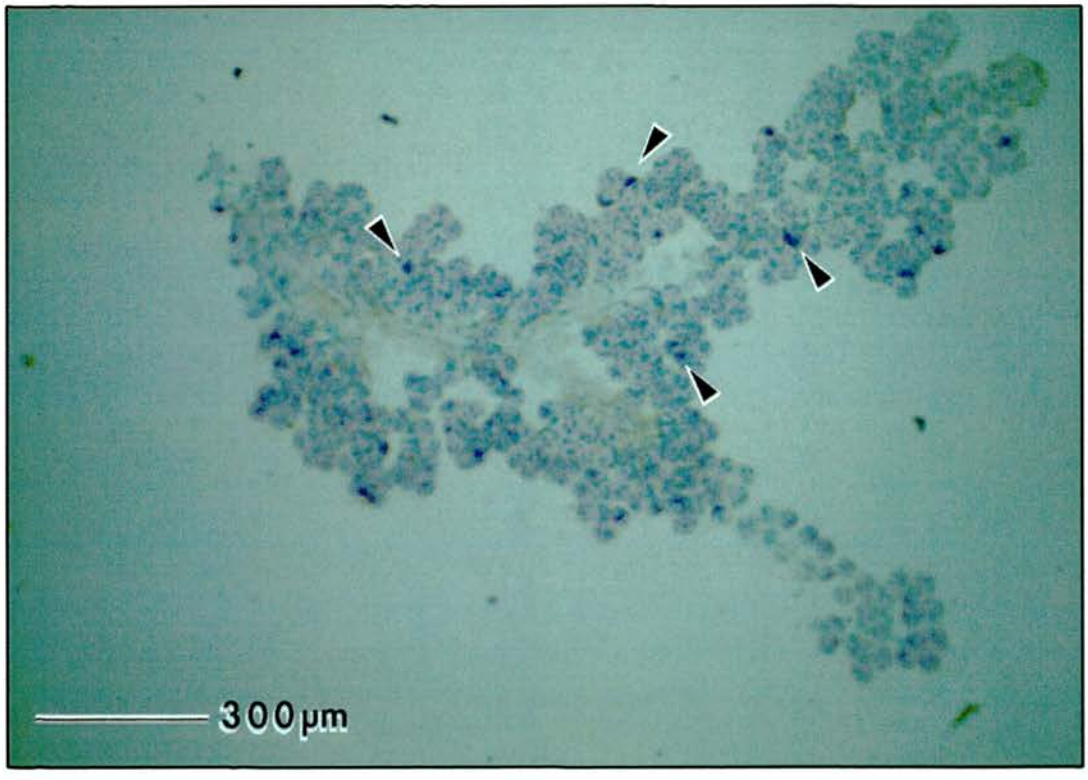
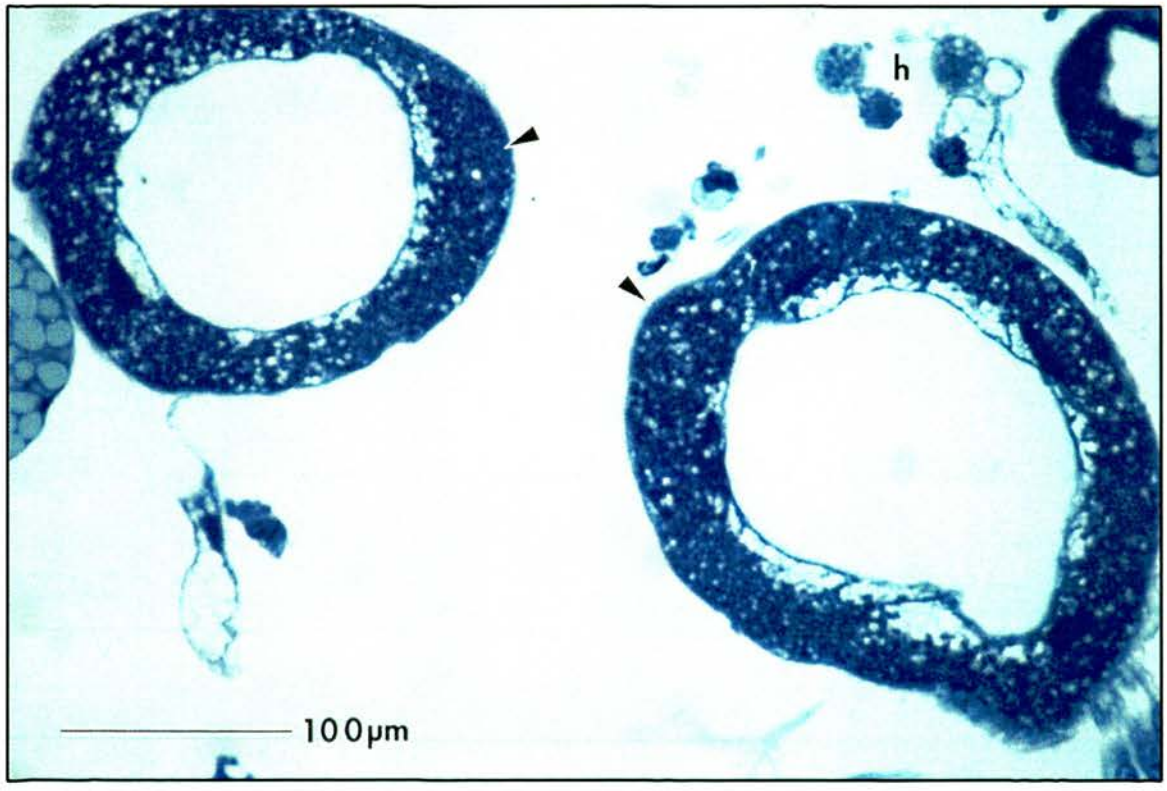

300µm

Figure 6.17a. Section through a Malphighian tubule from a day 21 post-detachment, *T. parva* infected adult. The arrow points to *Wohlbachia* (small blue dots) in the epithelial cells. The organisms are present in considerably smaller numbers than in control ticks. n – cell nucleus.

6.17b. Section through Malphighian tubules from a day 21 post-detachment uninfected, adult. The *Wohlbachia* (indicated by the arrows) are very densely packed into the epithelial cells. h – haemocytes.


20µm


h
100µm

15 ticks) in the tubules of infected ticks there appeared to be fewer and smaller clumps of intracellular *Wohlbachia* rickettsias than in the uninfected ticks (Figs. 6.17a and 6.17b).

Distorted legs

After completion of the moult, approximately 20% of the live adult ticks of both sexes from the batches that detached at the highest piroplasm parasitaemias were crippled with distorted legs. This was never seen in the uninfected ticks. Scanning electron micrograph images of the nymphal exoskeleton revealed large plaques which were blocking the internal opening of the coxae (Figs. 6.18a and 6.18b) in some infected ticks, causing the previously mentioned leg deformities. Increased magnification showed a dense carpet of unicellular and hyphal fungal growth (Fig. 6.19) which may have been responsible for causing the build up of the plaques.

Tick infection and mortality levels

Tick infection and mortality levels are displayed in Table 6.1. The table shows that the prevalence and abundance of infection in ticks decreased as the piroplasm parasitaemia in the calf increased. The mortality of the ticks was also very high at increased calf parasitaemias.

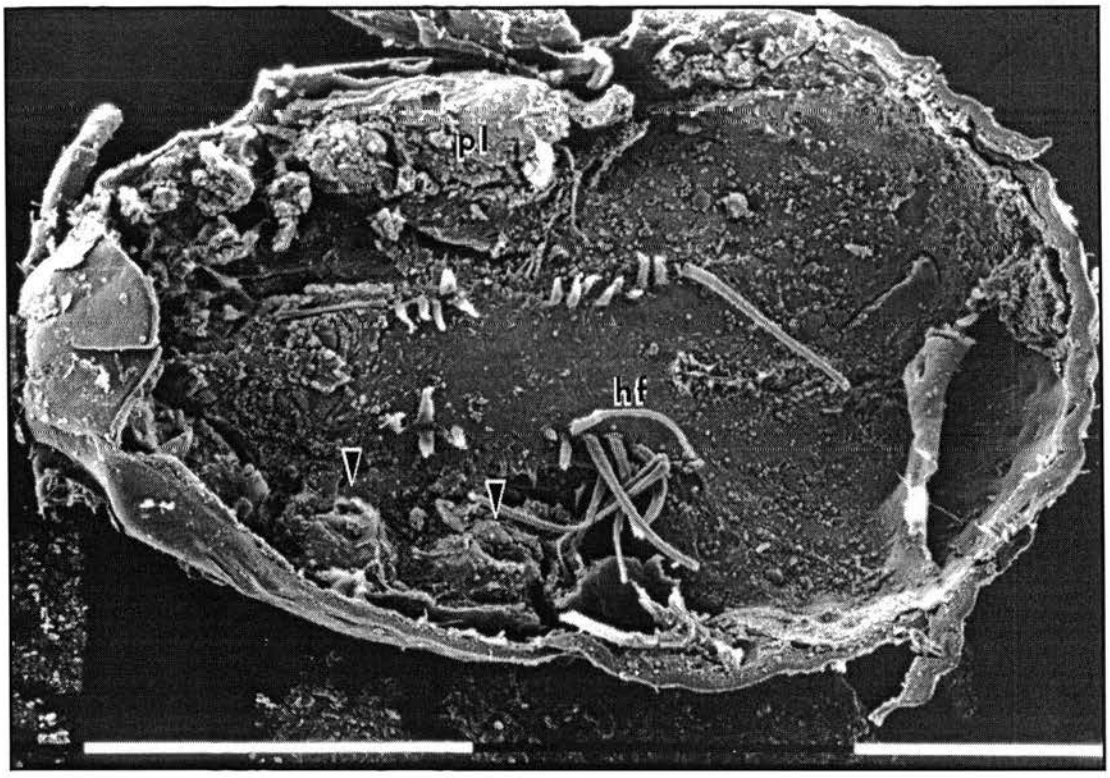
6.4. *Effect of* T. parva *infection on fecundity and hatch rate*

Stark differences were apparent between the infected and uninfected females in terms of their reproductive capacity (Table 6.2). Raw data is presented in Tables 4A and 4B, Appendix 4.

Uninfected, engorged females detached over a 3 day period from days 6 to 8 (after placement on the host). Infected females detached over a 4 day period from days 7 to 10. A two-tailed, Mann-Whitney test revealed an significant difference between the medians of the two populations with the infected ticks taking longer to engorge and detach ($P < 0.0001$, U-statistic = 472, U' = 1928).

Figure 6.18a. Scanning electron micrograph of the inside ventral surface of a *T. parva* infected, day 16 post-detachment nymphal exoskeleton displaying severe pathology. During the dissection process, developing adult with severe leg deformities was removed. The arrowheads point to structures that should be the internal openings of the coxae (through which the developing adult's legs should have grown). Long strands of hyphal fungi (hf) can be seen attached to the cuticle, which are thought to be involved in the development of large plaques (pl) which would have greatly restricted the legs of a developing adult. The white bars represent 1mm in length.

6.18b. Scanning electron micrograph of the inside ventral surface of a day 16 post-detachment nymphal exoskeleton from an uninfected tick. The unrestricted, internal openings of the coxae are clearly indicated by the arrowheads. The white bars represent 1mm in length.


pl
hf

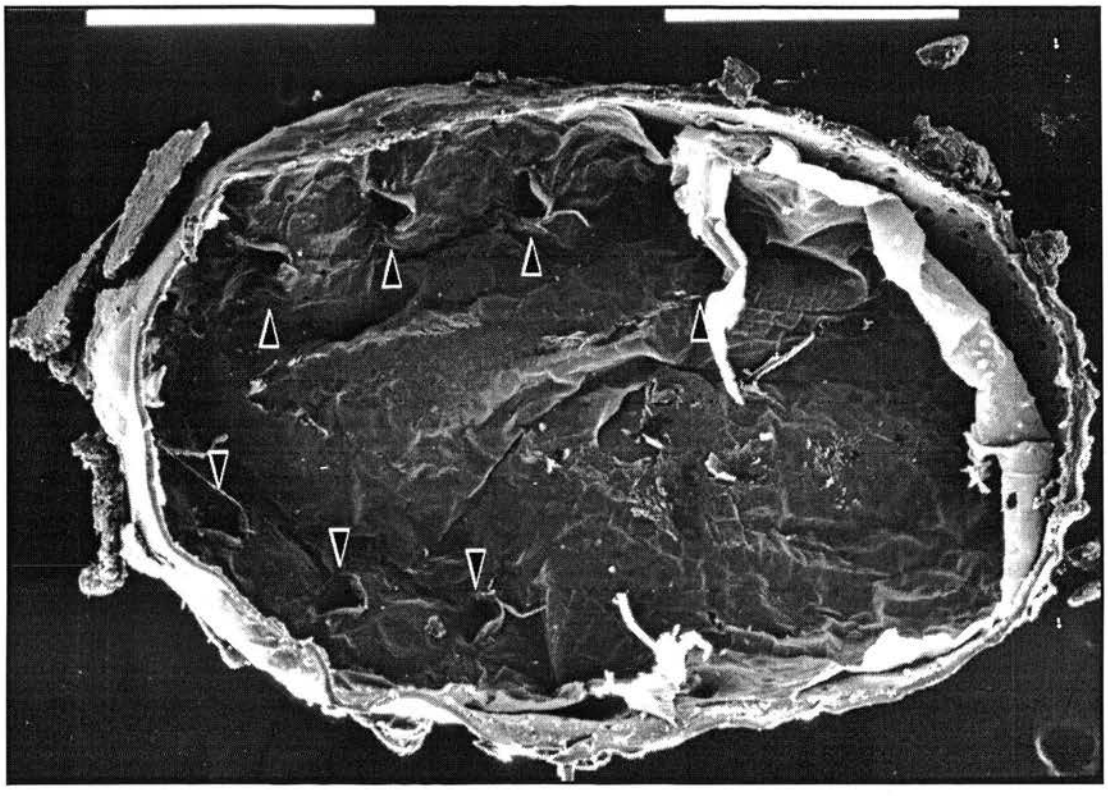
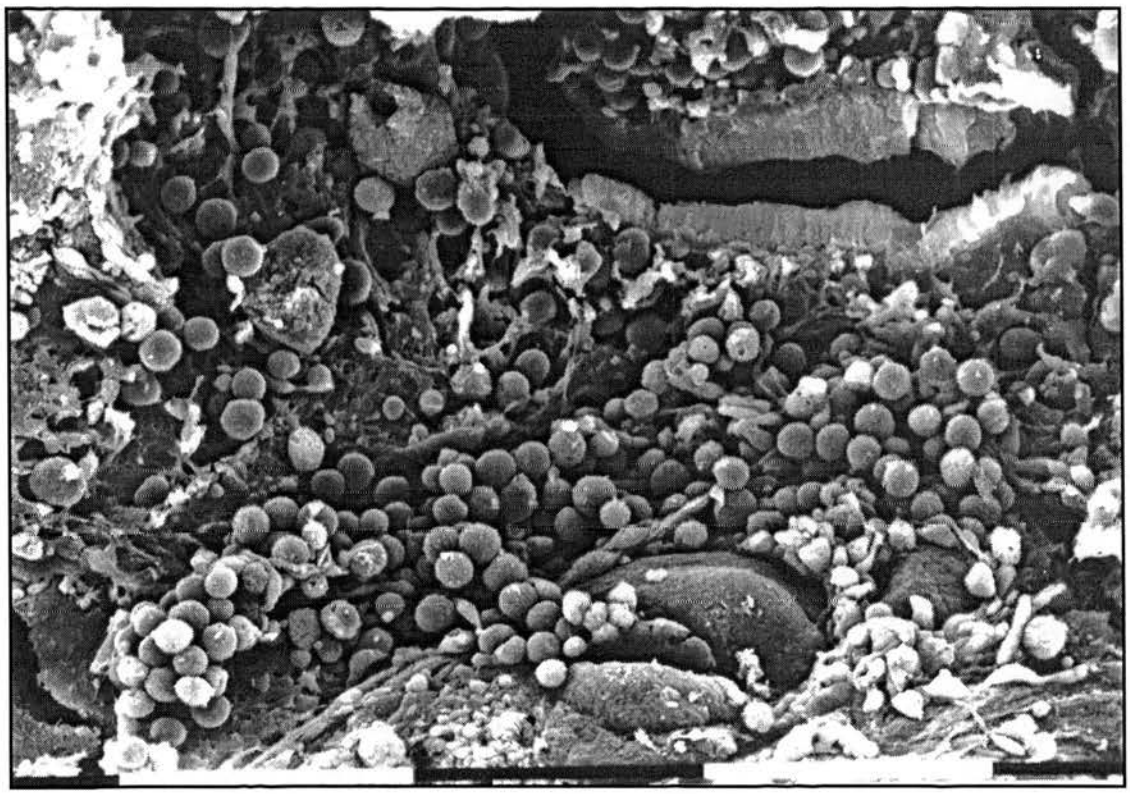


Figure 6.19. High magnification, scanning electron micrograph showing large numbers of microsporidia-like cells on the inside ventral surface of a day 16 post-detachment nymphal exoskeleton displaying severe pathology. The white bars represent 100μm in length.

Table 6.1. The piroplasm parasitaemia on the day of nymphal detachment was compared with the eventual adult sporoblast infections and the tick mortality data from each detachment day. The table clearly demonstrates the non-linear relationship between calf parasitaemia and tick infection levels. It also shows the high levels of mortality seen within the tick batches. n - the number of ticks sampled.

Table 6.2. The reproductive capacity of batches of uninfected and infected female ticks was compared. The parameters measured included; the time the females took to engorge and detach, their engorgement mass, egg batch mass and percentage hatch rate of the eggs. * the figure in brackets represents the mean percentage of egg batch mass compared to the initial female engorgement mass.



Piroplasm % on Detachment	Prevalence m (n) / f (n)	Abundance m / f	Mortality (%)
3.2 - 13.1	100 (19) / 100 (20)	129 / 129	5
13.1 - 19.6	70 (20) / 70 (20)	29 / 88	30
19.6 - 22.4	-	-	100
22.4 - 22.4	71 (14) / 74 (19)	11 / 13	75

Parameter	Uninfected	Infected
Feeding time (days)	6 - 8	7 - 10
Engorgement mass (mg)	436	298
Egg batch mass (mg)	281 (68%)*	185 (66%)*
Egg hatch rate (%)	61	12

The uninfected females imbibed larger bloodmeals than the infected ticks. The average weight of the uninfected ticks was 436mg compared to 298mg of the infected ticks. A two-tailed, unpaired t test revealed a significant difference between the means of infected and uninfected female engorgement weights ($P < 0.0001$, $t = 5.875$, 96 d.f). The egg batch weight of the uninfected ticks was 281mg compared to 185mg of the infected ticks. Once again, a two-tailed, unpaired t test revealed an extremely significant difference between the population medians ($P < 0.0001$, $t = 6.405$, 96 d.f). Infected tick engorged weights were 68% of uninfected tick weights and the infected ticks' egg batch weights were 66% of those of the controls. This represented an engorged body weight/egg batch weight ratio of 64% in uninfected and 62% in infected ticks.

There was a large difference between the numbers of hatched larvae. The uninfected ticks' egg batches had a mean hatch rate of 61% compared to 12% for the infected ticks' egg batches. A two-tailed Mann Whitney test revealed a significant difference between the medians of the two populations ($P < 0.0001$, U statistic = 363, U' = 1940).

Discussion

The physiology of infected and uninfected ticks was very similar up to day 8 post-detachment. This is not surprising when one considers the parasite development within the host at this time. Newly formed zygotes cross the gut wall very soon after the tick has engorged and detached. Electron micrograph images of this process in *Babesia* (Agbede *et al* 1984) suggest that the parasites cross the cell membrane and enter the cytoplasm without any obvious signs of cell damage. *T. parva* zygotes remained in the cytoplasm for about two weeks. In this time they increased in size enormously, but significant growth tended to occur just before the parasites were ready to transform into kinetes (see Chapter 5). In the first 8 days therefore, competition for nutrients would be the main effect ticks would probably have had to contend with. By day 9, there were slight irregularities in the form of lipid vesicles

seen in the infected cells and this may be the first signs of parasite induced effects. The moulting process started to show signs of inhibition at this point, possibly because the competition for energy resources was becoming more acute. The count data presented in Chapter 5 showed zygote and kinete numbers had increased enormously on this day.

Infected ticks started showing very clear differences from the controls by day 12. One of the most obvious differences seen in the sections was the distribution of gut contents. Control ticks displayed a very homogenous digestion, suggesting the contents were evenly digested throughout the entire gut. Infected ticks however, showed a large degree of heterogeneity in the distribution of vesicle bound digestive products within the gut. The dissimilarity between infected and uninfected ticks in this regard was such that their digestive processes must have been very different. Lack of haematin in the infected ticks' guts showed that bloodmeal digestion was proceeding at a much slower rate than in the controls. The variation in protein vesicle size seen in some of the infected ticks (Fig. 6.3a) suggests that digestive processes were uneven in different areas of the gut. The smaller vesicles nearer the gut wall suggest they were being digested sooner than the larger vesicles in the centre of the gut. The structure of the gut wall may have been damaged, such that the continual muscular contractions necessary for efficient digestion were insufficiently powerful to properly mix the gut contents, resulting in a heterogenous distribution of digestive products.

Malphighian tubules are very active throughout the moult, producing massive amounts of guanine excretion. The tumour-like, malformed tissue that occluded the vessel lumen was seen in a number of sections. The amount of digestive products found within the tubules could have resulted in blockage, forming an enormous build up of guanine within the tick. Although the huge rectal sacs filled with guanine were only seen after the ticks appeared to be displaying gross pathological effects (as opposed to being a root cause of them), the internal pressure caused by being unable

to expel the guanine is likely to have been a major contributor to the rapid morbidity and mortality seen in affected ticks.

By day 12, the batch of ticks used up to this point were considered to be so abnormal in their moult as to be incomparable with the equivalent day of moult in control ticks. From the parasite quantitation point of view (Chapter 5), it would have been very informative to have related the parasite numbers right the way through a tick series from a single detachment day. From a tick pathology perspective however, examining different batches including those that exhibited severe pathology (which led to 100% mortality) through to less affected ticks was very useful as a wide range of pathology was seen.

Vernick *et al* (1995) showed EM images of dead *Plasmodium gallinaceum* in a vacuolated gut epithelial cell of *Anopheles gambiae*. It was not known whether the pathology was as a result of a mosquito immune mechanism directed at the parasite or in response to release of toxins from the dying parasite. They described an amorphous area surrounding the parasite as a 'fuzzy zone' that was composed of finely granular and filamentous material. Normal endoplasmic reticulum profiles in other areas of the host cell suggested that pathological effects (at least up to the time of processing) were localised around the dying parasite. The mosquito strain used was refractory for the *Plasmodium* species and was known to kill the parasite within the intracellular environment of the gut epithelial cells. No information was given as to whether the host cell was destroyed as a result of parasite invasion. Destruction of gut epithelial cells would be extremely detrimental as it may allow digestive products to contaminate the haemocoel. The motile digestive cells seen in the haemolymph of day 15 ticks show this may have been the case for many of the guts which showed very high *T. parva* burdens, leading to destruction of the gut wall and consequent high mortality within the tick batches. Becker-Feldmann *et al* (1985) showed electron micrographs which revealed that damaged epithelial cells of *A. stephensi* infected with *P. yoelii nigeriensis* ookinetes were extruded from the regular gut epithelial layer. They stated that such damage could be replaced to a limited extent

by neighbouring cells or by cellular regeneration, but if the lesions were too numerous, the injury was not tolerated and the mosquitoes died.

A large number of surviving ticks which detached at high piroplasm parasitaemias and that experienced high mortality showed serious limb deformities. The presence of plaques, associated with fungal infection, appeared to be responsible by blocking the space available for the limbs to develop. The limbs were therefore restricted between the developing adult body and nymphal exoskeleton, resulting in their stunted and twisted appearance. The skin of most mammals supports populations of bacteria and fungi that cause no apparent ill-effects. The extended feeding time of ticks will expose them to considerable challenge from these potential pathogens. They are unlikely to cause any adverse effects to healthy, uninfected ticks because they will be digested and/or excreted along with the bloodmeal. Maier *et al* (1987) stated that *Plasmodium* ookinete damage to the mosquito midgut epithelium can also be exploited by other organisms, especially opportunistic bacteria and microsporidia. Lowenberger *et al* (1996) ran a series of experiments in which they investigated the *Aedes aegypti* immune response in relation to *Brugia malayi* infection. Mosquito haemolymph was inoculated with bacteria to activate the immune system and the effect on concurrent *B. malayi* infection levels was investigated. Infection was found to be lower compared with control and sham injected mosquitoes when fed on low to moderate microfilaraemias, but that the immunity broke down at high microfilaraemias because the immune system seemed incapable of suppressing the high parasite and bacterial populations. An analogous situation may have occurred with the deformed ticks. The salivary gland infections were not assessed in these particular ticks, but SEM images suggest that they supported dense populations of fungi. Large numbers of parasites, consequent damage from parasite invasion processes and the presence of bacteria and/or fungi may have been too much for the ticks to cope with, resulting in the gross pathology observed. The ticks affected by these deformities would have been incapable of questing to acquire a host as their movement was very limited.

Christensen *et al* (1989) showed an increase in haemocyte numbers in *A. aegypti* in response to *Dirofilaria immitis* microfilarial challenge. An increase in tick haemocyte numbers was shown in *Dermacentor andersoni* as a result of bacterial inoculation into the haemolymph (Johns *et al* 1998). Both methods used to count the cells were relatively sophisticated, involving radioactive labelling of the cells, followed by scintillation counter detection and phase contrast haemocytometry respectively. The haemocyte numbers did not look markedly different between the control and infected ticks in the histological sections examined in this study. However, the method was far from ideal in comparison with the above mentioned techniques and would merit further investigation in future transmission studies.

Wohlbachia are differentially distributed along the length of tick Malphighian tubules. Balashov (1972) reported that the rickettsiae are distributed along the entire length of the tubules in *Ixodes ricinus* and *Argas persicus*, while in *Rhipicephalus turanicus* and *Hyalomma asiaticum* (among others) they tended to be found in the distal half. The skewed distribution of the organisms in Rhipicephalid ticks makes accurate comparison of their numbers in the tubules of infected and uninfected ticks difficult with the techniques employed in this study. Serial sectioning of a whole tick would be the preferred method, and may be considered for future work. The sections examined for this study suggested a reduction in the numbers of *Wohlbachia* in the infected ticks. This is unsurprising considering the general health of the infected ticks, as they are likely to have represented a nutritionally poorer environment for the rickettsiae compared to the control ticks which were digesting their bloodmeal as normal.

The lack of pathological effects in Type I, II or IV (in male ticks only) acini, coupled with the concentration of pathological effects in e cells of Type III acini indicate that *T. parva* is highly selective of its target cell, and does not enter other types of cells less selectively and fail to survive in them. The pathology seen in the sections of the salivary glands was, in some cases, very severe and showed acini that were completely destroyed. This was even more evident when glands were dissected from

adult ticks for the infection assessment. Ducts completely bare of acini were commonly seen and this would have greatly compromised the feeding performance of affected ticks. Shaw and Young (1995) found *T. parva* induced pathology in *R. appendiculatus* salivary glands. The ticks had very highly abundances (> 200 infected acini) and a number of Type II acini were infected in addition to Type III. About 20% of infected Type II acini showed incomplete development and/or necrosis of the host cell and sporoblast and this suggested that the cells were an inappropriate environment for parasite survival and development.

Results from the fecundity and hatch success studies showed that *T. parva* infection had a very significant effect, not only on engorgement and egg batch weights, but also on eventual hatch rates. It is interesting to note that the weight of engorged female/egg batch weight ratios in the uninfected and infected groups were virtually identical (64 and 62% respectively). The difference in egg batch weights is directly correlated with the different engorgement weights of infected and uninfected females. Since infected ticks produced the same ratio of engorgement mass to egg batch mass as the controls, this suggests that the female ticks' reproductive capacity has not been adversely affected. Lack of egg hatching is indicative of infertile mating, which may indicate the reproductive organs of the male ticks had been affected by the parasites. The reduction in female engorgement weights may have been caused by two factors. Firstly, the guts of the infected ticks showed pathology and as a result, may have been unable to accommodate the same size of bloodmeal as the uninfected ticks, and secondly, the salivary glands showed pathological lesions with damaged and/or missing acini which were unable to function to their full capacity, thereby limiting the ticks' ability to feed. Although egg batch weights were reduced compared to the controls, a 100% hatch rate would still have collectively produced many thousands of larvae. A useful future experiment will be to mate uninfected male and female ticks with infected ticks and compare the levels of egg production and hatching. If *T. parva* infection did cause male tick sterility it could be a major factor in affecting tick population dynamics in ECf endemic areas.

CHAPTER 7

Detection of *Theileria parva* in *Rhipicephalus appendiculatus* using *in situ* hybridisation

The aim of these experiments was to design a highly specific and sensitive *in situ* hybridisation-based technique to detect *T. parva* in tissue sections of *R. appendiculatus*.

Introduction

A requirement for a more efficient method of detecting *T. parva* in *R. appendiculatus* histological sections was indicated by the quantitative results from the Giemsa's stained histology slides in Chapter 5. In these experiments, parasites were not detected between days 2 and 8 post-detachment. The apparent increase in zygote numbers, peaking on days 8 and 15 post detachment was believed to be representative of their increased detection as opposed to increased numbers. The technique could also be somewhat subjective and was very time consuming, making the development of a rapid, objective method a priority for future vector competence studies. An attempt was made to develop such a technique using *in situ* hybridisation of a *T. parva* specific probe.

Antibody-based approaches using reagents that could detect *T. parva* within tick sections by recognising the parasites' outer membranes would be preferred, because the membranes would be present in every tick section that contained a recognisable parasite. The difficulty of such an approach is that no reagents exist and so sera would have to be raised. *Theileria* undergoes enormous morphological and biochemical changes throughout its development within the tick (Friedhoff 1990). It is highly likely that the membrane characteristics of the different stages will also be extremely divergent, as in *Babesia* (Friedhoff 1990). An antibody raised against the membrane of one form would not necessarily react with that of another. Although polyclonal sera could be raised against the piroplasm and sporoblast and sporozoite forms with relative ease as they can be purified by well established protocols, and the gametes could probably be obtained by *in vitro* culture techniques, xygotes and

kinetes present a serious problem. Methods to obtain these stages by *in vitro* techniques or purifying them directly from ticks have not been reported and may be very difficult. For these reasons, an antibody-based detection system was not considered.

RNA or DNA *in situ* hybridisation or PCR offers a plausible alternative to an antibody approach. Using RNA as a target was rejected for the purposes of this study because it was considered important to be able to detect dead or dying parasites. This is of fundamental relevance to vector competence studies because anti-parasite mechanisms within the tick are of primary concern. RNA would be greatly reduced if not absent depending on the time of parasite death and the rapid effect of endogenous RNAses.

DNA *in situ* PCR was not thought to be necessary because of the high TPR1 sequence copy number within the *T. parva* genome. The degree of amplification following *in situ* gene amplification is very inefficient compared to solution PCR (Hoyland and Mee 1997). Solution PCR regularly undergoes up to 40 cycles in a typical amplification profile in which the temperature will rise to 94^0C at the template denaturing step. The limit to the number of these cycles usually depends on the presence, or at least the unacceptable build up of non-specific reaction products, or the depletion of reaction ingredients such as deoxynucleotidephosphates or *Taq* DNA polymerase activity. Forty cycles of heating and cooling would have a deleterious effect on the morphology of tissue sections however, and some researchers have found a maximum of 10 cycles is all that is required to achieve a positive result (Hoyland and Mee 1997). The use of *in situ* PCR was therefore felt to be unjustified on a genome with multiple copies of a target sequence because of the proportionally small increase in nucleic acid that would result weighed against the difficulties and costs of developing the PCR system over a straightforward hybridisation technique.

DNA *in situ* hybridisation was selected as the method to identify *T. parva* in infected *R. appendiculatus*. DNA is more stable than RNA and remains detectable even when the host organism is dead, as was demonstrated by detection of *T. parva* in tick faeces (Chapter 5).

Materials and methods

Prehybridisation steps

Fixation

The choice of tissue fixative is determined by the type of hybridisation and detection to be carried out subsequently. Precipitating fixatives such as ethanol or acetic acid provide good probe penetration but can result in poor retention of tissue morphology and a substantial loss of RNA (Hoyland and Mee 1997). They are most appropriately used for DNA:DNA hybridisation. Crosslinking fixatives such as (para)formaldehyde and gluteraldehyde provide better RNA retention but can reduce probe penetration by extensive protein crosslinking (Hoyland and Mee 1997). Fixation conditions have to be determined experimentally and depend on the required application and tissue type.

Tissue is fixed at 4^0C (to inhibit the action of endogenous ribonucleases) for varying lengths of time depending on its permeability and the nature of the fixative. The sample is then embedded in medium, usually paraffin wax, and prepared for sectioning. The embedding medium for histological samples acts to support the tissue during the sectioning procedure. The most commonly used method of preparing samples for hybridisation is to embed the material in paraffin wax and cut sections ranging in thickness from 5 to 10 μm. One disadvantage of paraffin wax sections is that light does not penetrate thicker sections as well as thin ones which decreases resolution. The morphology of specimens embedded in paraffin wax is also less well preserved compared to those embedded in and cut from methacrylate. Methacrylate, in common with other plastic resins, inhibits probe penetration through the section, limiting hybrid formation to the section face - referred to as 'surface only' labelling (Timms 1986, Kellenberger *et al* 1987). Methacrylate embedded material has therefore been used very little in hybridisation techniques. To overcome this problem, an acetone de-embedding procedure to remove the methacrylate from

semi-thin sections and allow greater probe access has recently been described (Warren *et al* 1998).

7.1. *Preparing control material*

Salivary glands used for positive control material were dissected from highly infected ticks (batches used in experiments described in Chapter 5) which had been incubated at 37^{0}C, 100% r.h for 5 days. The dissected glands were placed into Karnovsky's fixative for a minimum of two days at 4^{0}C. Salivary glands for negative control material were dissected from uninfected ticks (from the same original batch as the infected ticks) kept at 37^{0}C, 100% r.h for 5 days. Around 100 salivary glands were pooled and placed in small moulds containing molten 1% agarose in 0.5 x TBE. When the agarose solidified, it was removed from the moulds in preparation for paraffin wax embedding. The glands were easier to process when they were collectively contained in an agarose matrix. Staff in the Veterinary School Pathology Department processed the material for paraffin wax embedding. Water was removed from the samples overnight through a graded alcohol series and gradually replaced with liquid paraffin wax (Shandon Histoplast). The wax was kept at 45^{0}C to prevent it from solidifying. Liquid paraffin wax was then poured into a mould and the sample added and aligned as necessary. An embedding cassette labelled with the appropriate sample details was placed on top of the mould and became fixed in place as the paraffin wax solidified on a refrigerated plate. For section cutting, the blocks were cooled on a refrigerated plate and 5μm thick sections cut on a rotary microtome. The sections were floated onto water heated to 40^{0}C and allowed to flatten out before being picked up onto Poly-L-Lysine coated slides, prepared as detailed below.

7.2. *Slide preparation*

Microscope slides were thoroughly cleaned by placing them in a 1:1 ether/alcohol solution for 5 mins. After air drying, they were placed in a rack and immersed in 0.01% Poly-L-Lysine (Sigma) in distilled water for 5 mins. The rack was removed and the slides dried in an oven at 60^{0}C for 1 hr. Poly-L-Lysine solution aids section adherence to the slides.

Slides with histology sections were placed in an oven at 60°C from 1hr to overnight to increase section adhesion and melt the paraffin wax. When removed from the oven, they were immediately transferred to m-xylene for 3 x 5 min washes (to dissolve and remove the paraffin wax). Rehydration through a graded alcohol series followed with two 1 min washes in 100% ethanol, two 1 min washes in 95% ethanol, a 1 min wash in 70% ethanol and a 1 min wash in distilled water.

7.3. *Protease treatment*

The tissue was exposed to limited proteolytic digestion to increase target availability by partially digesting protein around the nucleic acid. The most commonly used protease is Proteinase K (PK), but successful use of pepsin (Emson and Gait 1992, Loos and Lichter 1996) and trypsin (Delvenne *et al* 1993, Kashima *et al* 1997) have also been reported. The optimum conditions for protease digestion must be determined empirically for each tissue, depending on fixation and probe types.

Proteinase K (PK) and trypsin were used to treat the tissue sections, with different concentrations being prepared in 0.1M Tris-HCl pH 8.0. The slides were placed on a heated block in a Hybaid Omnigene *in situ* PCR machine with a built-in humidified chamber to prevent evaporation. Around 100µl of solution was carefully applied over the sections and remained in place by surface tension. Slides were incubated at either 37°C or 42°C for time periods varying from 0.5 to 2.5 hr as described in Table 7.1.

Table 7.1. Proteolytic digestion conditions used on salivary gland sections for *in situ* hybridisation.

enzyme used	enzyme concentration	digestion time (hrs)	temp (°C)
Trypsin	10µg/ml	0.5, 1.0, 1.5, 2.0, 2.5	42
PK	5, 10, 15, 20, 30, 40, 60, 80, 100, 150, 200, 250, 300µg/ml	0.5, 1.0, 1.5, 2.0, 2.5	37, 42
PK	25mg/ml	1.0	42

7.4. *Re-fixation step*

The digestive enzyme step can result in a loss of morphological definition, but this can be improved by re-fixing the section in 0.4% paraformaldehyde. The low concentration of fixative is not thought to inhibit subsequent probe penetration to a significant extent.

Following protease treatment, the slides were washed in 1 x PBS for 3 mins and then post fixed in 0.4% paraformaldehyde at 4^0C for 5 or 15 mins.

7.5. *Probe preparation*

Double stranded DNA probes need to be denatured before use and are not as sensitive as single stranded DNA or RNA probes, but are simpler to produce and therefore, are frequently used. Probes that are too long i.e. over 500bp. are less sensitive because of access problems to tissue bound target sequences. Oligonucleotide probes can penetrate tissue that is difficult to access, for example, by over fixation of the tissue. A disadvantage with such short probes is the high background that can occur and the relatively small amount of label incorporation possible. For these experiments it was decided to use a PCR product (dsDNA) and an oligonucleotide probe.

Making the PCR product

The 402bp TPR1 PCR product was produced as described in Chapter 3.

7.5.a. Purification of the PCR products

PCR products to be labelled were purified to reduce background. A reaction tube containing TPR1 amplicon also contains unincorporated primers and deoxynucleotide tiphosphates, which need to be removed before labelling occurs. The amplified products were purified through Sephacryl® HR resin. Microspin™ S-200 HR Columns (Amersham Pharmacia Biotech) were used as they remove nucleic acid sequences ≤ 50 base pairs in size which will include primers and free nucleotides. Under centrifugation, large sequences pass between the resin beads and

out of the column in the eluate, whereas the much smaller primers and deoxynucleotide-triphosphates pass directly into the beads where they become trapped. The columns were prepared according to manufacturer's instructions. They were vortexed vigorously until the resin beads were homogenously mixed, the cap was loosened and the bottom closure snapped off. The column was placed in a 1.5ml microfuge tube and centrifuged at 735 **x g** for 1 min to remove excess TE buffer. The column was removed and placed into another microfuge tube. 100µl of amplified product was gently added without disturbing the beads and the tube was centrifuged at 735 **x g** for 2 mins to allow the amplicon to pass through the column and collect in the microfuge tube. Quantifiable amounts of amplified product produced by these methods allowed for accurate and reproducible labelling procedures.

7.5.b. Estimating PCR product concentration

It was essential to quantify the amplified product before probes were made. 1Kb DNA Ladder (Life Technologies Ltd) was used as a standard for establishing amplicon concentration. 10% of the total DNA in the ladder is known to be present in the 1636bp band (referred to as 1.6Kb band). The stock marker solution (1.0µg/µl) was diluted with sodium chloride to give a final salt concentration of 20mM NaCl. Loading dye (Sigma, England) was added according to manufacturer's recommendations. The dilution results in 3µl of marker solution containing 0.5µg of the 1.6Kb marker band. Different volumes of marker solution were loaded on a 1% agarose gel resulting in 0.25, 0.5, 1.0 and 2.0µg of the 1.6Kb band alongside dilutions of amplified product. The intensity and size of the bands from the amplicons were compared with that of the 1.6Kb bands, and a judgement was made as to the quantity of DNA in the amplified products. The intensity of fluorescence from ethidium bromide stained DNA depends on the length of the sequence, such that short probes will contain less label and will fluoresce less brightly than larger sequences. Therefore, it is likely that the amount of DNA in the 402bp. TPR1 band was slightly underestimated as it was compared with the 1.6Kb band in the marker.

7.5.c. *Fluorescein labelling*

Fluorescein can be used as a label for direct fluorescent detection. The strength of the signal can sometimes obscure details of cellular morphology however and as such, a direct fluorescent detection method was considered unsuitable for this experiment. The labelling method used however, utilised fluorescein as a hapten tag with an anti-fluorescein antibody conjugated with alkaline phosphatase for detection. The advantage of fluorescein over biotin (described in section **7.5.**4.) as a label is that it overcomes problems associated with endogenous biotin in the specimen which could lead to high background.

One of the labelling methods used for the double stranded DNA probe was random priming (Feinberg and Vogelstein 1983). Random hexanucleotide primers in the reaction mix bind to single stranded DNA (double stranded DNA is denatured first) at various points along its length. The reaction includes a labelled nucleotide and the Klenow fragment of DNA polymerase which produces sequences of mixed length (because of the varying annealing positions of the primers) with label incorporation along the length of the products. This is a particularly suitable method to label nucleic acid for *in situ* hybridisation because the varying probe lengths produced offer a wide range of options for specimen penetration. The range of probe lengths produced is advantageous if the tissue shows variable fixation, such that some target areas will permit access of larger probes than others (because of the different surrounding protein conformation for example).

7.5.c.1. Random prime labelling using fluorescein

The *Ready To Go* random prime labelling kit (Pharmacia Biotech) was used to label DNA. The reaction ingredients are incorporated in a hydrophilic bead which only lacks labelled deoxycytosinetriphosphate (dCTP). Fluorescein labelled dCTP (Fl-dCTP) (Pharmacia Biotech) was used in this experiment. The Klenow fragment of DNA polymerase in the reaction produces sequences of mixed length (because of the varying annealing positions of the primers) with fluorescein incorporation along the product. The manufacturer's protocols were followed throughout. The TPR1 DNA

solution (1.25µg - 15µl) was denatured by placing in a heated block at 95^{0}C for 5 mins and then placed directly on ice for 2 mins. The denatured amplicon was added to a *Ready To Go* tube containing one reaction ingredient bead (contains reaction buffer*, dATP, dGTP, dTTP, FPLC*pure*® Klenow Fragment (7-12 units) and random 9-mer oligodeoxyribonucleotides). Distilled water and Fl-dCTP (140µM final conc.) were added and mixed thoroughly to make a total reaction volume of 35µl. The tube was placed in a water bath at 37^{0}C for 2 hr to allow the random priming reaction to take place. The labelling reaction was stopped by storing the tube at -20^{0}C until required. *- the manufacturer did not specify the ingredients of the reaction buffer.

7.5.c.2. Confirmation of fluorescein labelling efficiency

The fluorescent properties of fluorescein were used in a rapid membrane based assay to check the success of the labelling reaction. A series of Fl-dCTP dilutions were prepared by diluting the 500µM stock solution in TE buffer (10mM Tris, pH 8.0, 1mM EDTA). 5µl volumes of 100µM, 20µM, 10µM, 5µM, 2µM, 1µM and 0.5µM were dotted onto a piece of dry Hybond N+ membrane (Amersham). 5µl of labelled probe and 5µl of 100µM Fl-dCTP solution were dotted onto a separate piece of Hybond N+. The liquid was absorbed into the membrane but was not allowed to dry completely. The membrane strip was then floated on prewarmed 2 x SSC at 60^{0}C and gently shaken for 15 mins to remove unincorporated nucleotides. The negative control should show little or no fluorescence. The membrane was then placed on a U.V transilluminator alongside the reference strip with the nucleotide dilutions. Viewing with U.V light allows a comparison of labelling efficiency with the reference strip. The intensity of the fluorescence emitted by the probe was compared to a range of standards which were prepared on a separate membrane by dotting a dilution series of the Fl-dCTP. The probe was considered to be adequately labelled if it showed fluorescence equivalent to 20µM to 2µM Fl-dCTP. The labelled amplicon was passed through a S-200 spin column (Amersham Pharmacia Biotech) to remove any unincorporated fluorescein.

7.5.d. *Biotin labelling the TPR1 amplicon*

Biotin is a natural growth factor in every living cell where it is found mainly bound to protein and polypeptides. It is particularly rich in tissue like liver, yeast and milk. One of its greatest benefits for use in research is the very powerful bond it forms with avidin (a protein found in egg white). This allows a non-antibody mediated detection system to be used which can be advantageous under certain circumstances, although anti-biotin antibodies are readily available. Nucleic acid labelling with biotin can be accomplished by chemical crosslinking, enzyme incorporation and energy from heat and/or specific wavelengths of light. The fact that the procedures are simple, inexpensive and produce very sensitive probes has made biotin one of the most commonly used haptens for nucleic acid labelling.

Photoprobe® Biotin from Vector Laboratories is a photoactivatable form of biotin. On exposure to heat or U.V light of 365nm wavelength, the biotin becomes highly reactive and very readily forms covalent bonds with single or double stranded nucleic acid.

7.5.d.1. Thermocoupling

The manufacturer's protocols (Vector Laboratories) were followed throughout. Labelling reactions wth varying concentrations of biotin were set up as well as a control reaction with water in place of biotin, as it is the biotin diluent. Concentrations of biotin used were: 1.4×10^{-3} M (stock conc.), 1.4×10^{-5} M, 1.4×10^{-7} M, 1.4×10^{-9} M. 40µl of Photoprobe Biotin (1mg/ml stock conc.) was added to 20µg of TPR1 amplicon in 40µl of TE buffer. 40µl of light mineral oil was overlaid on the reaction mix to prevent evaporation, and the reaction mix was incubated at 95^0C for 30 mins in a heating block to couple the biotin to the PCR product. After the coupling reaction, 80µl of 0.1M Tris, pH 9.5 was added and mixed thoroughly (the higher pH removes any unincorporated biotin by neutralising the H^+ group on the biotin moiety, thereby increasing the molecules' solubility). 160µl of 2-butanol was added (to remove water and concentrate the labelled probe solution) and the mixture was vigorously vortexed for 20 seconds. The phases were separated by centrifugation

at 13000 **x g** for 1 min and the upper 2-butanol phase containing unincorporated biotin was discarded. Another 160µl of 2-butanol was added and the process repeated to reduce the volume of DNA in buffer to approximately 40µl. The labelled probe was precipitated by adding 10µl of 10M ammonium acetate, 2µl of 1M $MgCl_2$ and 125µl of -20^{0}C ethanol, mixing thoroughly and incubating at -20^{0}C for 15 mins. The probe was pelleted by centrifugation at 13000 **x g** for 20 mins and then washed with 70% ethanol and centrifuged at 13000 **x g** for a further 3 mins. The pellet was resuspended in 40µl of TE buffer and were stored at -20^{0}C until required. The products from the reactions were run on a 1% agarose gel to compare the effects of the size of the resultant probe. Optimum labelling should result in a probe that is very similar to that of the native DNA in size (although it will be slightly bigger because of label incorporation). Higher biotin concentrations may overlabel the probes, which would result in decreased binding efficiency to complementary nucleic acid sequences. The larger molecules formed as a consequence of attached biotin would be slower to migrate through agarose gels than native DNA.

7.5.d.2. Dot blotting the thermocoupled biotin labelled probe

Running the probes in agarose gels indicated their molecular size but did not directly confirm that label incorporation had occurred. After the appropriate size had been confirmed by gel analysis, probes were dotted onto membranes so that label incorporation could be confirmed by enzyme based detection techniques.

Dilutions of TPR1 labelled probes, unlabelled amplicon (negative control) and biotin labelled DNA supplied with the biotin labelling kit (positive control) were prepared in 6 x SSC. The control labelled DNA was biotinylated λ *Hin*dIII DNA. The concentrations of TPR1 labelled probes were 5.0 x 10^{-2}µg/µl to 5.0 x 10^{-3}µg/µl, unlabelled amplicon was 5.0 x 10^{-2}µg/µl to 5.0 x 10^{3}µg/µl and the control labelled DNA was 6 x 10^{-2} ng/µl to 2 x 10^{-2} ng/µl. 5µl of each dilution was denatured at 100^{0}C for 3 mins, and then dotted onto Hybond N+ membrane. The probe mixture was allowed to dry and then the DNA was fixed to the membrane by exposure to 254nm UV light for 30 seconds. The membrane was washed three times in Tris

buffered saline (TBS - see Appendix 6) for 10 mins and was then placed in 1 x Casein blocking solution (Vector Laboratories - see Appendix 6) with agitation at 37^{0}C overnight. A 2.0 x 10^{-4} M solution of Alkaline-Phosphatase-Streptavidin (Alk-Phos-Strep) was prepared in 1 x Casein solution 30 mins before use. After the Casein blocking solution was discarded and replaced with the Alk-Phos-Strep solution the membrane was continually agitated in the hybridisation oven for 30 mins at room temperature followed by 3 x 10 min washes in TBS. The membrane was soaked in 0.1M Tris-HCL pH 9.5 for 5 mins and then overlaid with NBT/BCIP solution (Roche Diagnostics stock solution - see Appendix 6). The container was covered with aluminium foil to allow the colour reaction to develop in the dark. A dark purple colour usually developed within 20 to 30 mins, indicating successful probe detection. If the reaction was left to develop for a number of hours, the signal strength increased, but so did background staining over the whole membrane.

7.5.d.3. Photocoupling

The Photoprobe Biotin can also be photocoupled to the DNA probe. The protocol followed was the same as that described for the thermocoupling reaction, except that the tubes were placed in an ice bath with their lids open, rather than the heating block, and were irradiated with a hand-held U.V lamp (365 nm) placed 2cm above the tube rim for 30 mins. Subsequent probe precipitation was as described in Section 7.5.d.1., and the labelling was confirmed by dot blot detection as for the thermocoupled probe. Dilutions of labelled and unlabelled TPR1 amplicon were prepared in 6 x SSC and 1µl was dotted onto Hybond N+ membrane. The dilutions made were 0.1, 1 x 10^{-2}, 1 x 10^{-4} and 1 x 10^{-6} µg/µl.

7.5.d.4. Hybridisation of biotin labelled probe to control DNA (photocoupling reaction)

Probes were initially run in agarose gels to establish they were the correct size. They were then attached to membranes and detected using enzyme based colour reactions to confirm label incorporation had taken place. The final step in validating the probes was to use them to detect complementary DNA sequences which had been attached

to membranes. This revealed whether label incorporation interfered with the nucleic acid binding sites on the probe. Dilutions of TPR1 amplicon, extracted *T. parva* DNA, extracted *R. appendiculatus* DNA and biotin were prepared in 6 x SSC and are shown in Table 7.2.

Table 7.2. DNA concentrations used for dot blot detection by biotin labelled probes.

Sample	concentrations
TPR1 amplified products	0.1, 5 x 10^{-2}, 1 x 10^{-2}, 1 x 10^{-3} µg/µl
T. parva extracted DNA	1.4 x 10^{-2}, 7 x 10^{-3} µg/µl
R. app extracted DNA	1.4 x 10^{-2}, 7 x 10^{-3} µg/µl
Biotin	1.4 x 10^{-3} M, 1.4 x 10^{-5} M

5µl volumes of each were denatured by placing at 100^{0}C for 3 mins and then placed immediately on ice for 2 mins. 1µl of each solution was dotted onto a piece of Hybond N+ membrane, air dried and fixed with a U.V cross-linker as described previously (section 7.5.d.2.). Prehybridisation solution (50% formamide, 5 X SSC, 2% Blocking Reagent, 0.1% sodium-laurylsarcosine, 0.02% SDS) was added (20mls per 100cm^2 membrane) and the membrane was agitated in a hybridisation oven for 1hr at 42^{0}C. The prehybridisation solution was discarded and replaced with 5 mls of hybridisation solution containing 60ng of probe. Hybridisation was carried out at 42^{0}C overnight. The membrane was washed twice at room temperature in 2 x SSC, 0.1% SDS (w/v) for 5 mins each time and then twice at 68^{0}C in 0.5 x SSC, 0.1% SDS (w/v) for 15 mins each time. Overnight blocking in 1 x Casein solution was carried out at 37^{0}C. The membrane was covered with a 2 x 10^{-4} M solution of Alk-Phos-Strep in 1 x Casein (which was made 30 mins before use) and incubated with agitation for 30 mins at room temperature. Three 10 min washes with TBS pH 7.5 at room temperature were carried out and the membrane was soaked in 0.1M Tris-HCl pH9.5 for 3 mins. It was then covered with NBT/BCIP solution (Roche Diagnostics

stock solution - see Appendix 6) and allowed to develop in the dark for 30 mins. A final wash in water was carried out before the membrane was air dried and the labelling intensities analysed to show probe hybridisation efficiency and specificity.

7.5.e.1. *Digoxygenin labelling*

PCR incorporation of label into the probe was also used. In this method, conventional PCR amplification was carried out, except that a proportion of one of the nucleotides was labelled. As the amplification reaction proceeds, label incorporation occurs as a consequence of template extension.

The protein label Digoxygenin (Roche Diagnostics), known as DIG, can be incorporated into a probe during the routine process of PCR. Digoxygenin (DIG) is a steroid isolated from *Digitalis* plants. As the blossoms and leaves of the plant are the only natural source of Digoxigenin, anti-DIG antibodies have a very low chance of binding to other biological material making it a very specific detection system. DIG can be very efficiently incorporated into nucleic acids by chemical linking and a variety of enzymatic procedures. PCR is carried out as normal except for the incorporation of DIG-dUTP in the reaction mix. dUTP can be substituted for dTTP although it is not incorporated with quite the same level of efficiency. A compromise concentration is established which allows an efficient amplification. The advantage of PCR label incorporation is that large amounts of highly labelled probe can be generated quickly.

7.5.e.2. DIG labelling using PCR

The TPR1 PCR product probe was also labelled with Digoxygenin using the method of PCR incorporation. Reaction mixes were set up containing 2mM Tris-HCl, pH 8.55, 5mM $(NH_4)_2SO_4$, 100µM EDTA, 1mM 2-mercaptoethanol, 0.05% Thesit™, 5% glycerol (contained within Thermometic Reaction Buffer), 200µM deoxyadenine-triphosphate (dATP), 200µM deoxyguanosinetriphosphate (dGTP), 200µM deoxy-cytosinetriphosphate (dCTP), 130µM deoxythyminetriphosphate (dTTP), 70µM DIG-deoxyuraciltriphosphate (DIGdUTP), 1µM each primer, 2.5mM $MgCl_2$, *Taq*

DNA polymerase (1.25u/µl). Reactions were set up with 175ng, 100ng and 30ng of DNA to compare the efficiency of the labelling procedure. A separate reaction was set up without DIG-dUTP to compare the amplification efficiencies. Reactions were overlaid with 50µl of light mineral oil (Sigma) to prevent evaporation. The cycling parameters were: one initial denaturing cycle at 95^{0}C for 2 mins followed by 30 cycles at 94^{0}C for 1 min, 65^{0}C for 1 min and 72^{0}C for 1 min. A final one cycle extension time of 72^{0}C for 10 mins was used. Following amplification, the PCR products were run in 1% agarose gels. Incorporation of Dixogygenin results in an apparent increase in molecular weight of the PCR product compared to the product produced in the absence of DIG-dUTP.

7.5.e.3. Labelling oligonucleotides with DIG

An alternative strategy to produce DIG-labelled amplicon probes was to label oligonucleotides using a DIG Oligonucleotide 3' End Labelling protocol (Roche Diagnostics). The TPR1 specific oligonucleotides IL194 and IL197 were used in the reactions. Terminal Transferase was used to incorporate DIG-dUTP onto the 3' end of the oligonucleotides. 100pmol of each primer was placed into a 0.2ml microfuge tube on ice, along with 1 x reaction buffer (25mM Tris-HCl, 0.2M potassium cacodylate, 0.25mg/ml bovine serum albumin, pH 6.6), 5mM cobalt chloride, 2.5 units/ml terminal transferase in a final reaction volume of 20µl. The mixture was incubated at 37^{0}C for 15 mins and then the reaction was stopped by the addition of 1µl 0.2M EDTA, pH 8.0.

7.5.e.4. Dot blotting DIG labelled probes

The labelling efficiency was examined by dot blotting DIG labelled probes. DIG labelled TPR1 probe (0.1µg/µl), DIG labelled 1Kb marker (2µg/ml) (positive control) and unlabelled amplicon (0.1µg/µl) (negative control) were diluted in 6 x SSC. After 5µl samples were denatured at 100^{0}C for 3 mins and immediately placed on ice for 2 mins, 1µl of each was dotted onto Boehringer Mannheim's Nylon Membrane and air dried. They were fixed to the membrane by exposure to UV light as previously described (Section 7.5.d.2.). The membrane was then soaked in

washing buffer (see Appendix 6) for 1 min in 1% (w/v) Blocking Reagent (Roche Diagnostics) in TBS, pH 7.5 and gently agitated for 1 hr at room temperature. Anti-DIG-Alkaline Phosphatase antibody was diluted in blocking buffer at 1:5000 dilution to give a working concentration of 150mU/ml. The blocking solution was discarded and the membrane was covered with the antibody solution for 30 mins on a gently rocking platform at r.t. The antibody solution was then discarded and the membrane was washed twice in washing buffer for 15 mins each wash at r.t. NBT/BCIP (Roche Diagnostics stock solution - see Appendix 6) solution was prepared in detection buffer (0.1M Tris-HCl, pH 9.5) according to manufacturer's instructions. The membrane was first equilibriated in the detection buffer for 2 mins before the detection solution was added. The developing reaction was carried out in the dark for 0.5 - 1 hr.

7.5.e.5. DNA detection with the DIG labelled probe

T. parva and *R. appendiculatus* DNA samples were diluted into 6 x SSC. Samples used were TPR1 amplified products (0.1µg/µl), *T. parva* cell culture DNA (14ng/µl), *T. parva* infected tick DNA (20ng/µl), *T. parva* infected blood DNA (2ng/µl), *R. appendiculatus* DNA (20ng/µl) and bovine DNA (2ng/µl). The samples and membrane were prepared as described previously (Section 7.5.d.2). The membrane was covered with prehybridisation solution (20 mls per $100cm^2$) and incubated at 42^0C for 2hr with agitation in a hybridisaton oven. DIG labelled TPR1 amplicon solution was denatured at 100^0C for 10 mins and then placed directly on ice for 2 min. 1µg of the probe was added to 10ml of hybridisation buffer (Section 7.5.d.4.). The prehybridisation solution was discarded and the hybridisation solution added for overnight incubation at 42^0C with agitation in a hybridisation oven. The hybridisation solution was then removed (and retained for reuse) and the membrane washed twice in wash solution at r.t. for 5 mins each wash. The detection protocol was as described above (Section 7.5.d.2.).

7.5.e.6. *PCR and membrane blotting with DNA extracted from fixed salivary gland material*

To investigate whether the DNA within the fixed material had been damaged or lost through processing, PCR were carried out in combination with dot blot detection. DNA was extracted from a large number (around 50 to 70 glands) and a small number (10 to 20 glands) of salivary glands as well as from material in paraffin wax sections. The salivary glands were taken directly out of Karnoxsky's fixative, washed in three changes of 1 x PBS and placed in 180µl of tissue lysis buffer (Buffer ATL, QIAgen). The paraffin wax sections were applied to slides, dewaxed with xylene and then scraped off with a sterile scalpel blade before or after 30 mins of 20µg/ml Proteinase K treatment at 42^{0}C. The tip of the scalpel blade was placed directly into the lysis buffer to remove the tissue. A new scalpel blade was used for every section to prevent cross contamination. The positive control DNA for PCR was extracted from unfixed salivary glands dissected from five *T. parva* infected ticks (10 individual glands) from the same batch used to prepare the positive control material for paraffin wax embedding. The ticks were incubated at 37^{0}C, 100% r.h for 5 days prior to dissection.

DNA was extracted from all of the samples using the QIAgen DNA extraction kit as described previously (Chapter 3). The DNA from each sample was subjected to TPR1 PCR amplification and the resulting products were run on agarose gels as previously described (Chapter 3). The products were also dotted onto a membrane for detection with a DIG labelled TPR1 probe.

7.5.e.7. *Prehybridisation solution*

A prehybridisation incubation is sometimes carried out to prevent or decrease background staining. The prehybridisation step is usually carried out at the same temperature as the hybridisation and in identical buffer with the exception of the probe. The tissue becomes equilibriated with components of the hybridisation buffer and potentially non-specific binding sites are blocked by constituents such as Denhardt's solution and unlabelled DNA (eg. herring sperm). A prehybridisation step

was not carried out in initial experiments, but was considered for inclusion if background was a problem.

7.6. Hybridisation steps

Hybridisation mix

Denhardt's solution containing bovine serum albumin (BSA) is used as a blocking agent to reduce non-specific binding of probes to proteins, polysaccharides and nucleic acids. Dextran sulphate is used as a reaction accelerator. It is strongly hydrated in aqueous solution; thus macromolecules have no access to water which causes an apparent increase in probe concentration and consequently higher hybridisation rates (Lathe 1985). Sonicated salmon or herring sperm DNA is added with the probe in order to act as a competitor against repetitive simple sequence stretches which may occur. Salt is added in the form of sodium chloride or trisodium citrate and acts to control the stringency of probe binding to the target sequences. Low stringency conditions involve high salt concentrations that allow non-specific annealing of probe, but may provide strong, specific reactions. High stringency conditions (low salt concentrations) reduce non-specific interactions but may reduce specific signal intensity. The appropriate salt concentration for a particular target-probe combination is determined experimentally. Formamide is a denaturing agent that destabilises double stranded nucleic acids by disrupting the hydrogen bonding. This allows lower temperatures to be used for optimal probe reannealing, which is of critical importance as it preserves cellular morphology as well as section loss from the slide.

Hybridisation temperature

Hybridisation temperature is determined empirically by the size of the probe, but is generally around 37 to 45^{0}C. Increasing the temperature increases the stringency and results in less non-specific hybridisation, but may reduce the signal intensity slightly. Hybridisation can be carried out under stringent conditions to reduce the chance of non-specific binding, followed by low stringency washing steps. Alternatively, hybridisation can be carried out under low stringency to encourage maximum probe

adherence, followed by high stringency washing steps. Low stringency hybridisation and low stringency washing conditions were used in these experiments. If background became a problem, it was planned to increase the stringency of the washing conditions.

Control slides were sampled at various points throughout the hybridisation procedures and stained for 15 mins in 5% Giemsa's stain to assess the condition of the tissue morphology.

In situ hybridisation procedure

40µl of hybridisation mix containing 50% deionised formamide, 1 x Denhardt's solution, 10% (w/v) dextran sulphate, 0.2 mg/ml sonicated herring sperm (Promega), 4 x SSC, 40ng DIG labelled TPR1 probe or 0.2pmole/µl (final conc.) DIG labelled oligonucleotide probes was overlaid onto the tissue sections. Minor variations were made to the hybridisation solution in some experiments as follows. The hybridisation mix (containing the TPR1 probe) was heated at 95^{0}C for 30 mins to size fractionate the probe before applying to the sections. Probe concentrations were doubled. Salt concentrations were doubled by increasing the amount of SSC (final conc. 8 x SSC). Addition of small volumes of 0.5M EDTA to give a final conc. of 1 x 10^{-2} M.

Following the addition of hybridisation solution, the slides were heated to 95^{0}C for 5 mins and then placed directly on ice for 2 mins. A piece of coverslip sized Parafilm (American Can Corp.) was applied (the side in contact with the backing paper on top of the hybridisation solution) to the sections to minimise evaporation and they were incubated at 37^{0}C or 42^{0}C overnight.

7.7. *Post hybridisation washing*

Post hybridisation washes are necessary to remove any unbound probe and they also serve to remove any non-specifically bound probe that may have a weaker interaction with non-complementary sequence. The stringency of the washes is controlled by the concentration of salt and the wash temperature.

The slides were placed in 2 x SSC at r.t to loosen the coverslips. Washing was then carried out in either two 5 min washes in 2 x SSC (r.t) and two 10 min washes in 0.5 x SSC (r.t, 37^{0}C or 42^{0}C) or in 2 x 5 min washes in 4 x SSC (r.t) and 2 x 10 min washes in 2 x SSC (r.t, 37^{0}C or 42^{0}C). A 2 min rinse in 0.3% (w/v) Triton X-100 in water was followed by a 3 min wash in TBS. The Triton X-100 step was omitted in later experiments to see if this resulted in positive hybridisation signals (as eliminating detergent reduces the stringency of the reaction).

7.8. *Detection procedures*

Detection of labelled probe is preceded by a blocking step to reduce background. Most blocking solutions contain bovine serum albumin or serum, although some suppliers produce their own protein based blocking reagent. The hapten detection reagent is applied to sections, usually diluted in the blocking buffer. An enzymatic reaction with a colour producing substrate commonly follows to allow detection of the signal. Counterstaining is performed at this stage to allow discrimination of surrounding material. Because hybridisation involves detection of RNA or DNA, the actual signal detection will occur only in cytoplasmic or nuclear sites and no detail of cellular structure outwith those areas is revealed. A counterstain which allows good morphological definition and adequate contrast with the hapten label is necessary to see the signal in the proper cellular context. Levamisole is sometimes added to the detecting buffers because it inhibits endogenous phosphatases. This is of use only with alkaline phosphatase detection systems.

1% Boehringer Mannheim Blocking Reagent in TBS was prepared by constant stirring on heated block. The slides were placed in the solution for 15 mins (r.t) and then very briefly rinsed in TBS. The anti-DIG antibody conjugate was diluted 1:5000 in the blocking solution to give a working concentration of 150mU/ml. 40µl was pipetted onto each section and incubated in a humidified chamber for 1 hr at room temperature. Two 10 min washes in TBS were followed by a 5 min equilibration in 0.1M Tris-HCl (pH 9.5) detection buffer. After air drying the slides 40µl of NBT/BCIP solution (Roche Diagnostics stock solution - see Appendix 6) was

applied. The slides were incubated in a humid chamber in the dark at room temperature for periods ranging from 0.5 to 24 hr, and then the slides were washed in TBS and mounted (50mM Tris-HCl pH 9.5 in 66% glycerol), ready for microscopic examination.

Results

7.9. *Preparing control material*

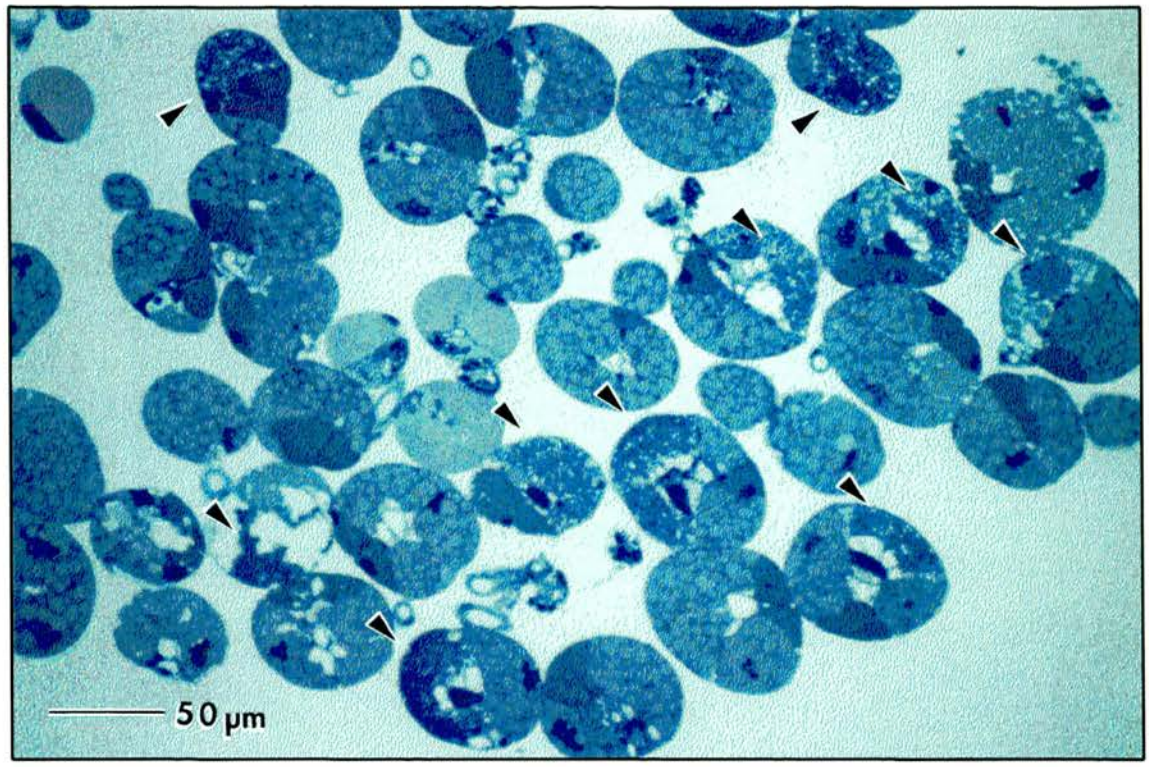
Figs. 7.1a and 7.1b show Giemsa stained sections through salivary glands representing negative and positive control material. As can be seen from the positive control, there are numerous sporoblasts and large areas that would allow probe annealing in *in situ* hybridisation procedures.

7.10. *Protease treatment*

The morphology of the sections were barely altered by any of the Proteinase K steps up to the level of 300µg/ml (data not shown). Figs. 7.2a and 7.2b show Giemsa stained sections from an infected salivary gland that was subjected to 25mg/ml Proteinase K digestion for 1hr at 42^0C during the *in situ* hybridisation procedure. The morphology was degraded compared to an unprocessed section (Figs. 7.1a and 7.1b) but not as poor as would have been expected from such a concentrated solution of Proteinase K, as total destruction of the section was anticipated. The cellular structure of the salivary glands duct and acini can be distinguished as well as mature sporoblasts. The efficacy of the Proteinase K was checked by using it to extract DNA from unfixed tick tissue using the QIAgen DNA extraction kit. The manufacturer's supplied protease was exchanged for the Proteinase K used in the course of the *in situ* hybridisation experiments. All material was efficiently digested and the extracted DNA was successfully amplified in PCR reactions (data not shown) which suggested the enzyme was fully functional.

Figure 7.1a. A section through *Theileria parva* infected salivary glands typical of that used as positive control material for *in situ* hybridisation. The arrows point to sporoblasts and indicate that there are many areas on the section which *T. parva* specific probes could potentially hybridise with.

Figure 7.1b. A section through uninfected salivary glands typical of that used as negative control material for *in situ* hybridisation.


50 µm

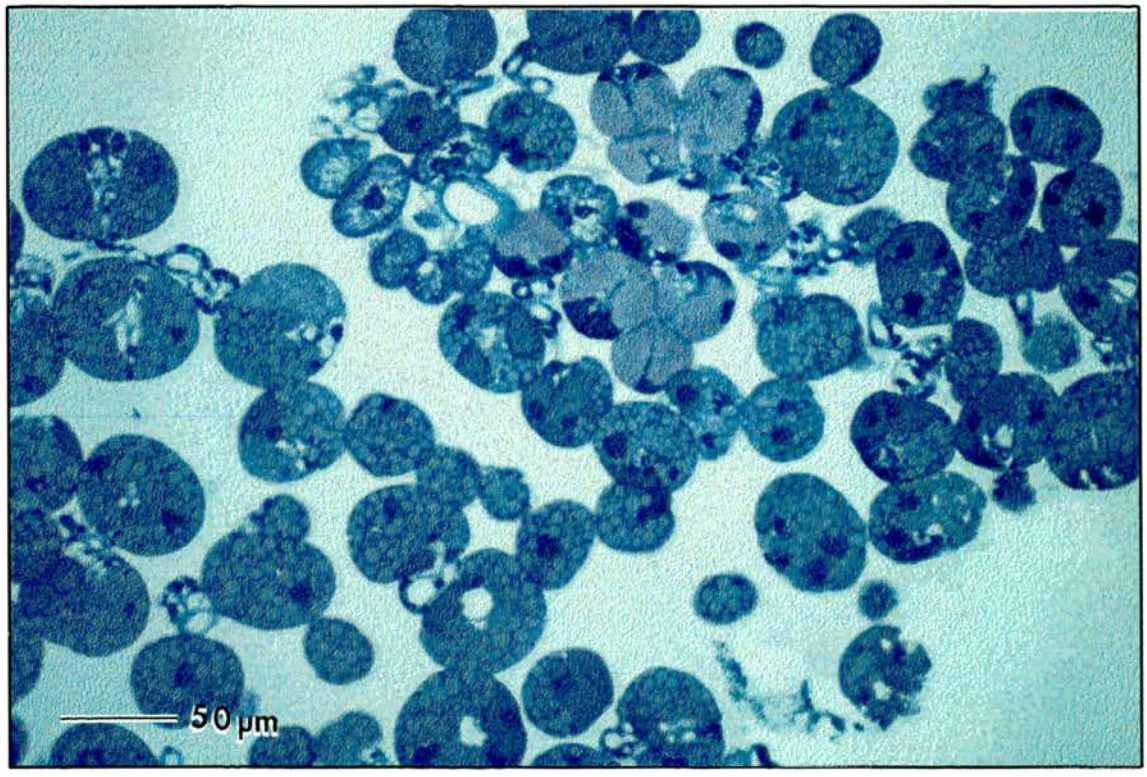
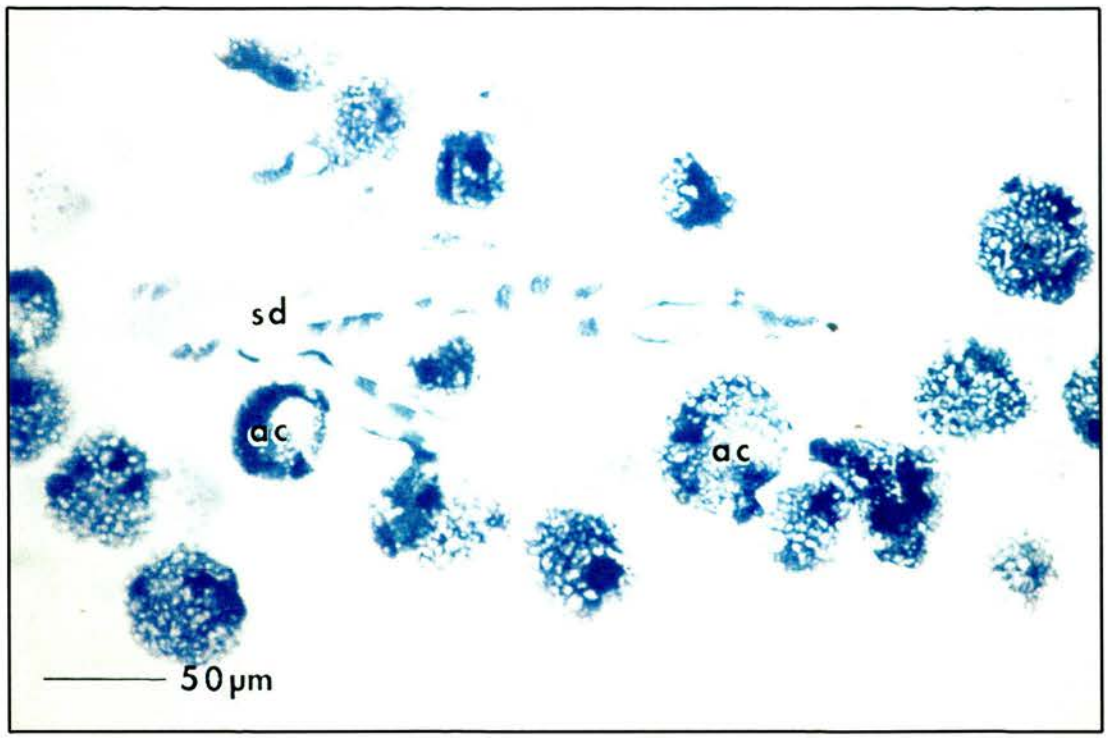

50 µm

Figure 7.2a. A *T. parva* infected salivary gland section exposed to proteolytic digestion with 25mg/ml Proteinase K. sd - salivary gland duct, ac – acini.

Figure 7.2b. Higher magnification view of a *T. parva* infected salivary gland section exposed to proteolytic digestion with 25mg/ml Proteinase K. Sporoblasts are indicated by the arrows. sd - salivary duct.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ac
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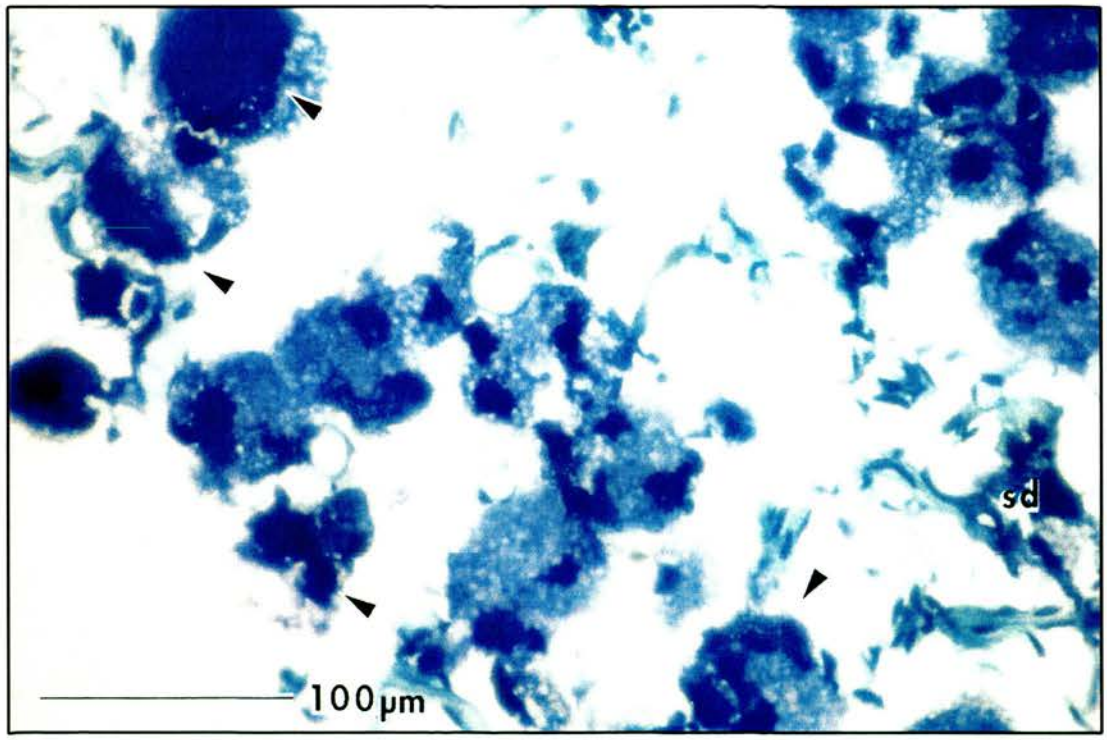
sd
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7.11. *Probe preparation*

7.11.a. Purification of the PCR products

Fig. 7.3 shows a comparison between TPR1 PCR product before and after passage through a Microspin column. There was a slight reduction in amplicon intensity. The size exclusion effect of primer and free nucleotide removal would be difficult to demonstrate on an agarose gel.

7.11.b. Estimating PCR product concentration

Fig. 7.4 shows a typical quantification gel. The intensity of the 1.6Kb marker band containing 500ng of DNA was judged equal to that of 12µl PCR amplicon solution. The PCR band was twice the size of the marker band (measured from the photograph), therefore 12µl of PCR product = ~1µg DNA.

7.11.c. *Checking the fluorescein labelling efficiency*

Dotting the labelled probe onto a nylon membrane to compare with the standards revealed the probe had incorporated very little fluorescein, as the spot was equivalent to a level between 1/500 and 1/1000 nucleotide dilution (data not shown).

Filtering the probe through a S-200 Microspin column revealed that the vast majority of the fluorescein was retained in the filter, showing it was unincorporated (Joos and Lichter 1996). The filter strongly fluoresced under U.V light whereas no fluorescence was seen in the TPR1 eluate solution (data not shown).

The use of fluorescein was discontinued after this point, as the TPR1 amplicon was not labelling.

7.11.d. *Biotin labelling the TPR1 amplicon*

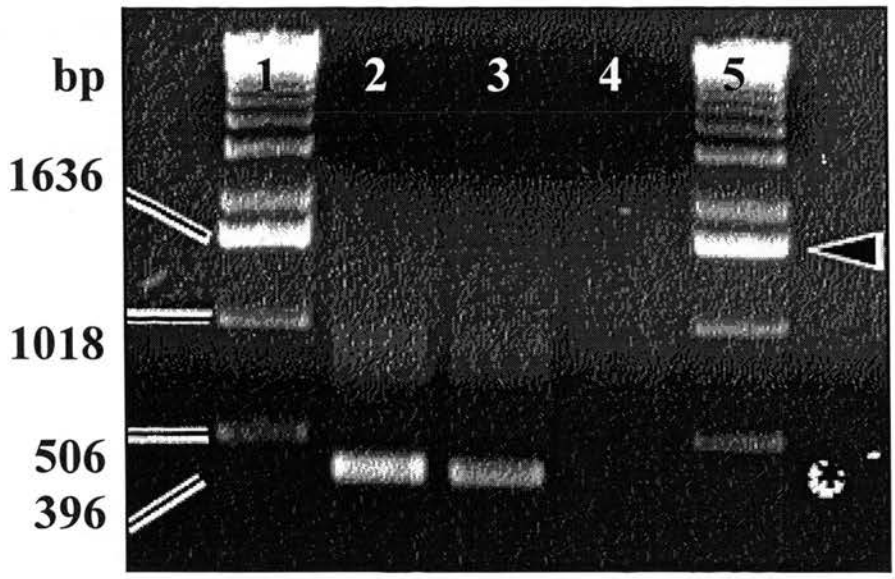
7.11.d.1. Thermocoupling

A quantitation gel was prepared as has been described previously. Fig. 7.5 shows the result. The probe did not migrate from the wells. The DNA size markers migrated through the gel as normal showing that the nature of the gel matrix was unlikely to be

Figure 7.3. Gel showing native TPR1 amplicon run beside amplicon that has been passed through a nucleotide size exclusion column. The arrow indicates the level of the 1636bp marker band which is generally referred to as the 1.6Kb band, and was used for quantifying PCR products by simple comparison of Ethidium bromide fluorescence intensity. The asterisk indicates the correct size of the 402/405bp *T. parva* gene PCR products. The molecular weight marker was used consistently in all the gels in this chapter, therefore the band sizes indicated are relevant to all three gels on this page. † refers to Sephacryl HR S-200 Microspin column (Pharmacia Biotech). [Lane] **1** - 1Kb marker. **2** - 5µl PCR product. **3** - 5µl PCR product (S-200)†. **4** - Negative (no DNA) control. **5** - 1Kb marker.

Figure 7.4. Gel showing a quantitation assay with dilutions of PCR products run alongside dilutions of 1Kb marker. The arrowheads indicate the 1.6Kb band with which the intensity of the PCR product bands was compared. The asterisks indicate the correct size of the 402/405bp *T. parva* TPR1 gene PCR products. [Lane] **1** - 0.25µg 1.6Kb marker band. **2** - 1.5µl PCR product. **3** - 0.5µg 1.6Kb marker band. **4** - 3.0µl PCR product. **5** - 1.0µg 1.6Kb marker band. **6** - 6.0µl PCR product. **7** - 2.0µg 1.6Kb marker band. **8** - 12.0µl PCR product.

Figure 7.5. A quantitation gel prepared with dilutions of thermocoupled, biotin labelled probe alongside dilutions of 1Kb marker. The arrowheads indicate the 1.6Kb band with which the intensity of the labelled probe was compared. The asterisk indicates the position of the probe. The probe had not migrated out of the loading wells, indicating a problem with the labelling technique. [Lane] **1** - 0.25µg 1.6Kb marker band. **2** - 0.75µg biotin probe. **3** - 0.5µg 1.6Kb marker band. **4** - 1.5µg biotin probe. **5** - 1.0µg 1.6Kb marker band. **6** - 3µg biotin probe. **7** - 2.0µg 1.6Kb marker band. **8** - 6µg biotin probe.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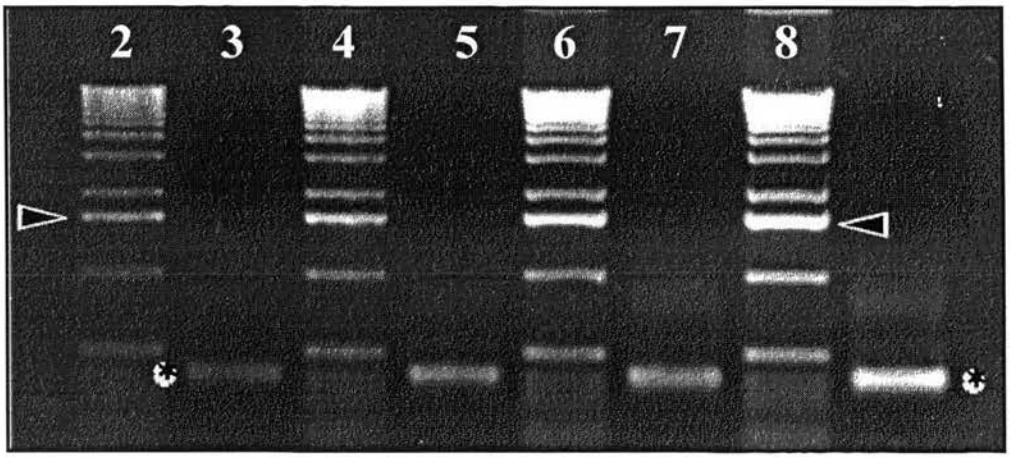
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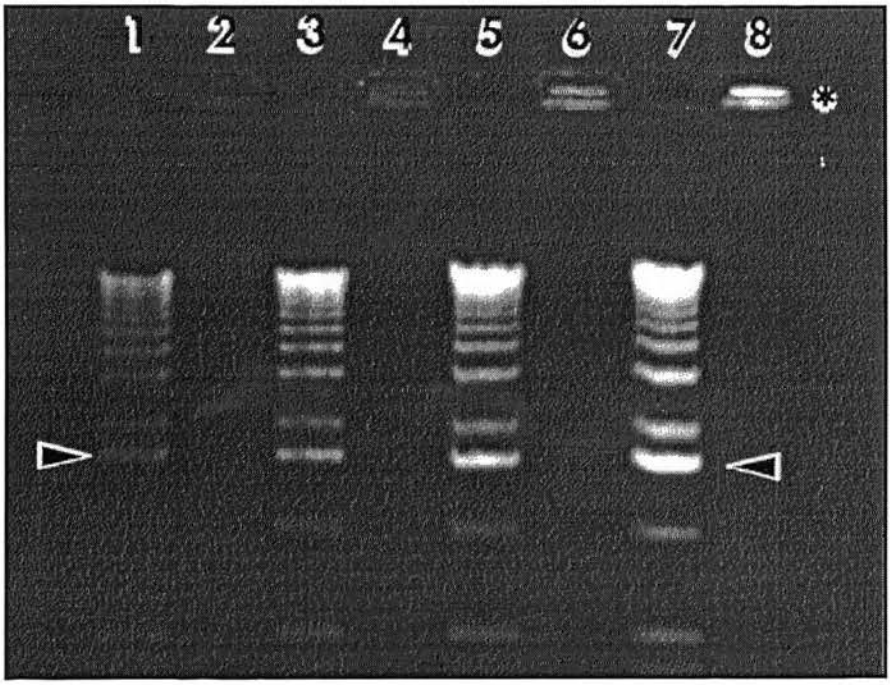
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at fault. Biotin incorporation would be expected to increase the size of the probe molecule, but not to the extent suggested by the products in Fig. 7.5.

7.11.d.2. Dot blot detection with the biotin labelled probe (thermocoupling technique)

The probe was attached to a membrane and detected with alkaline-phosphatase detection system to ensure the bands seen in the wells (Fig. 7.5) were actually DNA. Fig. 7.6 shows the TPR1 amplicon had been successfully labelled.

7.11.d.3. Varying the biotin concentration within the thermocoupling reaction mix

Fig. 7.7 shows results from the experiment with different dilutions of biotin added to the labelling reaction. If biotin overlabelling was responsible for the very large molecules seen in Fig 7.5 it would be expected that a size grading effect would be seen with varying concentrations of biotin included in the reaction mix. All of the TPR1 samples subjected to the heat treatment were unable to migrate from the wells, even the reaction with water rather than biotin. Biotin itself was not seen on the gel. It appears from this experiment that the heat treatment involved in the coupling reaction, and not the biotin itself was responsible for the effects seen.

The use of the thermocoupling technique was discontinued after this point as the heating procedure was altering the probe sizes, making them unsuitable for *in situ* hybridisation procedures.

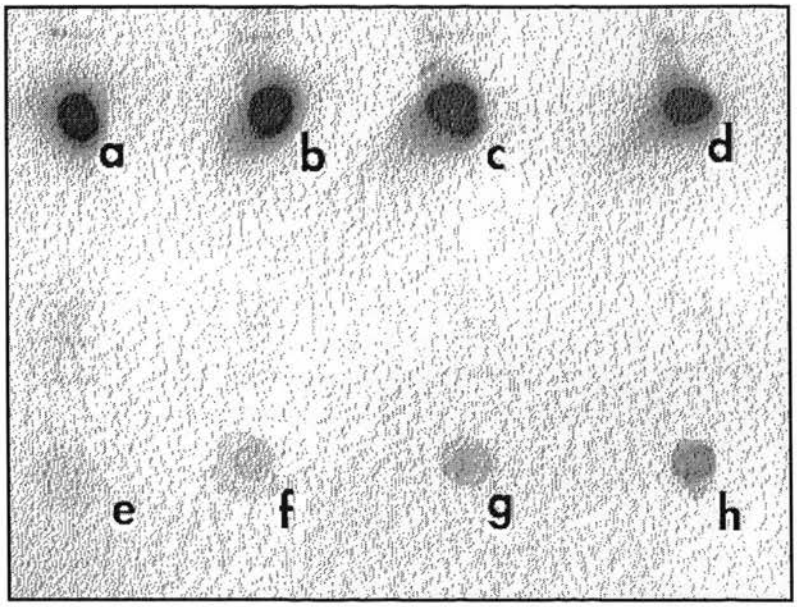
7.11.d.4. Photocoupling

The agarose gel results from the photocoupling reactions are shown in Fig. 7.8. The probes were all of the expected size (~ 402/405bp) and there was only a slight loss in band intensity which may be accounted for by incorporated biotin masking the ethidium bromide fluorescence from the nucleic acid.

Figure 7.6. Dot blot showing alkaline phosphatase-based detection of biotin labelled probe. **a**: 0.5μg biotin probe. **b**: 1μg biotin probe. **c**: 1.5μg biotin probe. **d**: 2μg biotin probe. **e**: 0.5μg TPR1 amplicon. **f**: 1.5μg TPR1 amplicon. **g**: 0.1ng control probe*. **h**: 0.3ng control probe. *The control probe was supplied with the PhotoProbe Biotin kit (Vector Laboratories). It was biotinylated λ *Hin*dIII DNA at 0.1ng/μl in TE pH 8.0.

Figure 7.7. Gel showing the results from varying the concentration of biotin in the thermocoupling reaction to investigate whether biotin was overlabelling the probes. A standard amount of TPR1 amplicon was used in each reaction with biotin concentrations ranging from 1:1 to $1:10^6$ dilution to determine whether probe sizes would be affected. The probes (pr) were still unable to migrate from the loading wells as can be seen, suggesting that the biotin problem is independent of biotin concentration in the labelling reaction. The arrowhead indicates the 1.6Kb band with which the intensity of the labelled probe was compared. The asterisk indicates the position of the probe. ‡ this represents the stock concentration supplied. † refers to Photoprobe Biotin (Vector Laboratories). [Lane] **1** - 1Kb marker. **2** - 5μg PCR product. **3** - 5μg PCR product + water. **4** - 5μg PCR product + 1.4×10^{-3} M‡ biotin†. **5** - 5μg PCR product + 1.4×10^{-5} M biotin. **6** - 5μg PCR product + 1.4×10^{-7} M biotin. **7** - 5μg PCR product + 1.4×10^{-9} M biotin. **8** - 5μl 1Kb marker.

Figure 7.8. A quantitation gel showing varying concentrations of unlabelled TPR1 PCR product were run alongside equivalent concentrations of photocoupled biotin labelled probes. The arrowheads indicate the 1.6Kb band with which the intensity of the labelled probe was compared. The asterisk indicates the position of the probe. [Lane] **1** - 0.25μg 1.6Kb marker band. **2** - 0.75μg PCR product. **3** - 0.75μg biotin probe*. **4** - 0.5μg 1.6Kb marker band. **5** - 1.5μg PCR product. **6** - 1.5μg biotin probe. **7** - 1.0μg 1.6Kb marker band. **8** - 3μg PCR product. **9** - 3μg biotin probe. **10** - 2μg 1.6Kb marker band. **11** - 6μg PCR product. **12** - 6μg biotin probe.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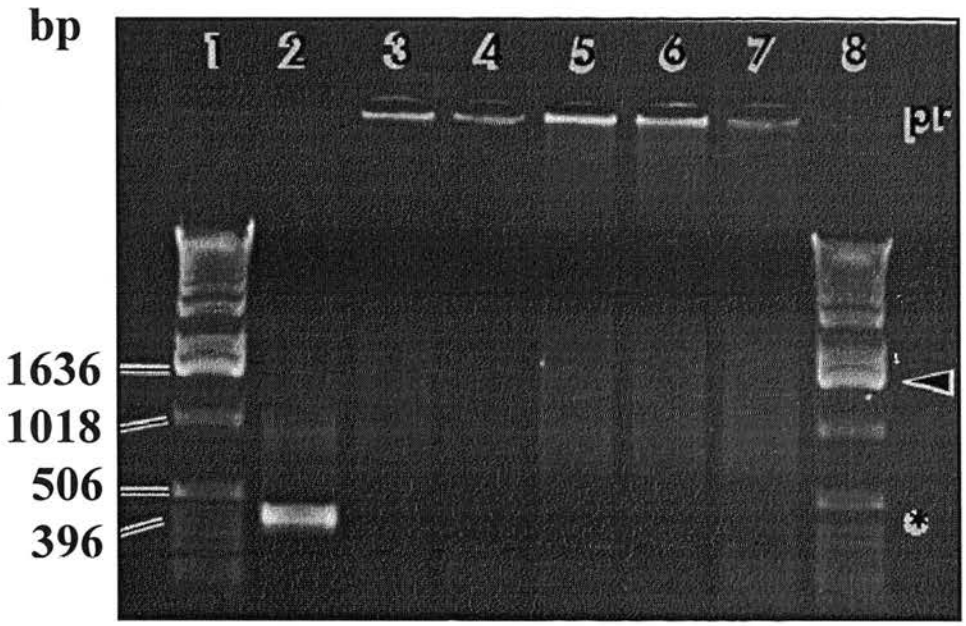
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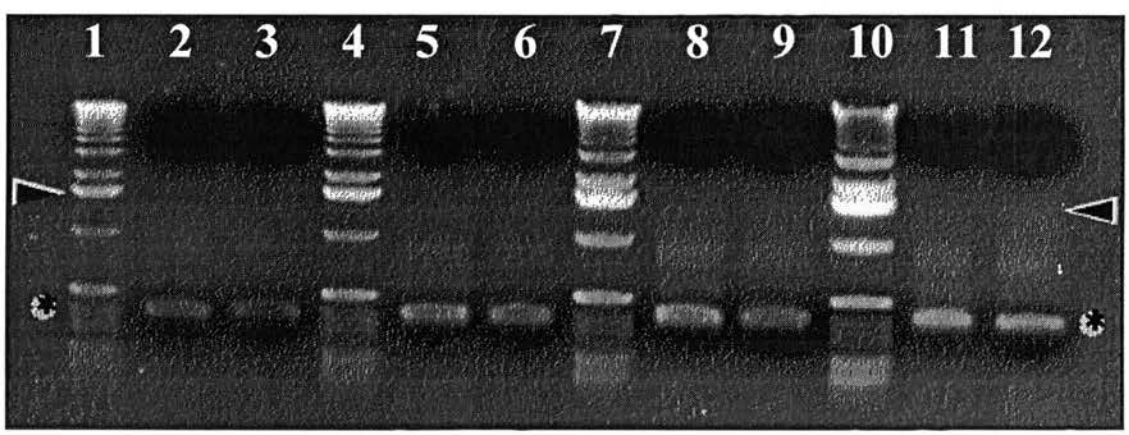
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7.11.d.5. Dot blot detection with the photocoupled, biotin labelled probe

The photocoupled biotin labelled probes were successfully labelled, with membrane detection possible at 5 x 10^{-2}µg. None of the unlabelled PCR product concentrations were detected (data not shown).

7.11.d.6. Hybridisation of biotin labelled probe to control DNA (photocoupling reaction)

None of the DNA or biotin samples were detected on the membrane (data not shown).

The use of photocoupled biotin was discontinued after this point as the TPR1 amplicon was labelling, but would not function as a probe.

7.11.e. *DIG labelling*

7.11.e.1. PCR incorporation of DIG

Fig. 7.9 shows the result of the amplification reaction. The DIG labelled probes appeared to be of equivalent intensity to the unlabelled TPR1 amplicons, suggesting the labelled nucleotides do not appear to have inhibited the reaction efficiency. The band sizes were very slightly larger with the DIG incorporation which is what would be expected (as DIG incorporation results in a larger molecule).

7.11.e.2. Dot blotting the DIG labelled probe

The amplicon was successfully labelled by DIG, as demonstrated by signal detection on a membrane. The signal was very intense, showing there was high label incorporation. No signal was obtained for the unlabelled, amplified products (data not shown).

7.11.e.3. DNA detection with the DIG labelled probe

Fig. 7.10 shows the dot blot DNA detection results. TPR1 amplified products were detected, *T. parva* cell culture DNA was very faintly recognised and a slightly

stronger signal was produced from DNA extracted from *T. parva* infected blood. None of the other DNA samples showed any signal detection.

7.11.e.4. Labelling oligonucleotides with DIG

Oligo labelling was successful with high probe incorporation. Dot blot detection of probes attached to the membrane revealed they were highly labelled. The oligoprobes detected the same range of amplified and non-amplified *T. parva* DNA samples on a dot blot as did the DIG labelled TPR1 probe shown in Fig. 7.10 (data not shown).

DIG labelling was made the label of choice for *in situ* hybridisation because it proved consistently successful with membrane detection of amplified product and native DNA preps.

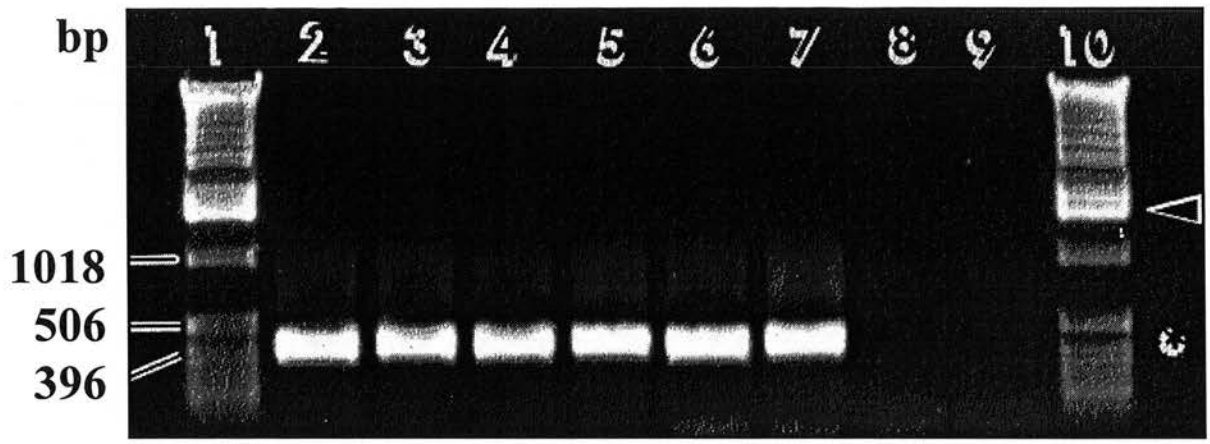
7.11.e.5. *PCR and membrane blotting with DNA extracted from fixed salivary gland material*

The DNA in the salivary glands was undoubtedly affected by the fixation procedure. Amplification did occur from DNA extracted from the large collection of *T. parva* infected salivary glands, but the amplicon was very weak compared to that of the positive control. The same is true of the amplification reaction from the small collection of infected glands where the band is only very faintly apparent. The positive control DNA was extracted from 10 unfixed, individual salivary glands and as can be seen from Fig. 7.11, the amplicon is very intense. The effect of Proteinase K on the sections could not be assessed because none of the DNA from treated or untreated tissue sections amplified. The dot blot results did not prove any more sensitive than the gel results. This may represent a reflection on the limitations of the dot blot sensitivity. However, considering the faint PCR amplicons produced from the large numbers of whole, infected salivary glands, it is likely that there was so little *T. parva* DNA in the sections that even after PCR, detection was impossible. Only the positive control, the large number of infected, fixed salivary glands and (very faintly) the small number of infected, fixed salivary glands were detected (data not shown).

Figure 7.9. Gel showing varying concentrations of *T. parva* DNA added to PCR with and without Dig-dUTP to compare the reaction efficiency of PCR labelling amplicons. 10µl volumes were run in the gel from each reaction. The quantity of DNA refers to the amount added to each 50µl PCR. The DIG labelling reactions were very efficient and the reaction products were very slightly larger due to DIG incorporation into the nucleic acid. The arrowheads indicate the 1.6Kb marker band and the asterisk indicates the 402/405bp. size of the PCR products. [Lane] **1** - 1Kb marker. **2** - 0.25µg PCR products. **3** - 0.25µg DIG probe. **4** - 0.75µg PCR products. **5** - 0.75µg DIG probe. **6** - 1.25µg PCR product. **7** - 1.25µg DIG probe. **8** - Negative (no DNA) control. **9** - /. **10** - 1Kb marker.

Figure 7.10. Various DNA samples (PCR amplified and non-amplified) were dotted onto a membrane and hybridised with a DIG-labelled *T. parva* TPR1 probe. The concentrations of DNA have not been given because a number of samples contain host and parasite mixed DNA. Quoting the nucleic acid concentration of these could be misleading. **a**: TPR1 PCR product. **b**: TPR1 PCR products. **c**: *T. parva* Muguga cell culture DNA. **d**: *T. parva* Muguga infected tick DNA. **e**: *T. parva* Muguga infected blood DNA. **f**: *R. appendiculatus* DNA. **g**: Bovine DNA.

Figure 7.11. Gel showing PCR results from DNA extracted from 5µm thick sections of *T. parva* infected salivary glands and uninfected glands. DNA was also extracted from whole, small (10-20) and large (50-70) collections of *T. parva* infected and uninfected salivary glands. The amplicons from lanes 3 and 6 represent a PCR from an equivalent number of salivary glands, except DNA from the glands in lane 3 was extracted from Karnovsky's fixed material compared to the unfixed glands in lane 6. The arrowhead indicates the 1.6Kb marker band and the asterisk indicates the 402/405bp PCR product. [Lane] **1** - 1Kb marker. **2** - large (50-70) collection of infected glands. **3** - small (10-20) collection of infected glands. **4** - large (50-70) collection of uninfected glands. **5** - small (10-20) collection of uninfected glands. **6** - +ve control: 10 unfixed, infected glands. **7** - /. **8** -ve control.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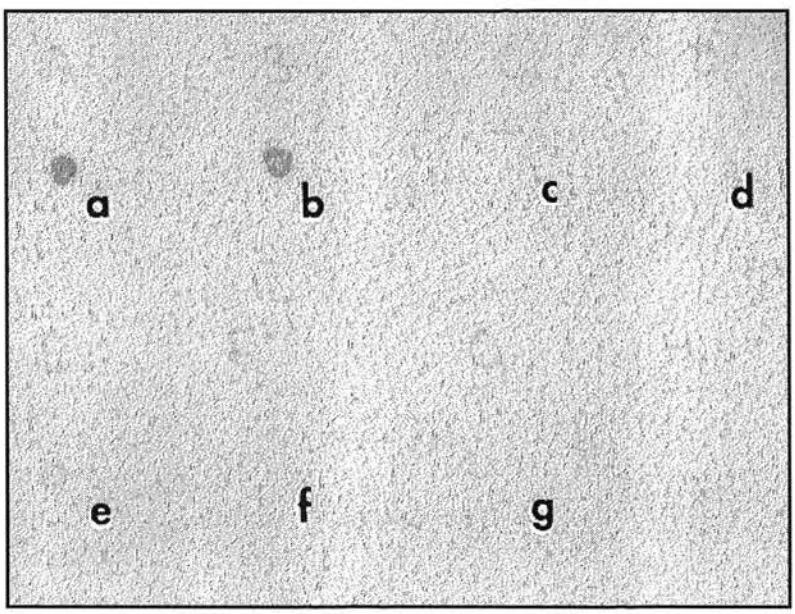
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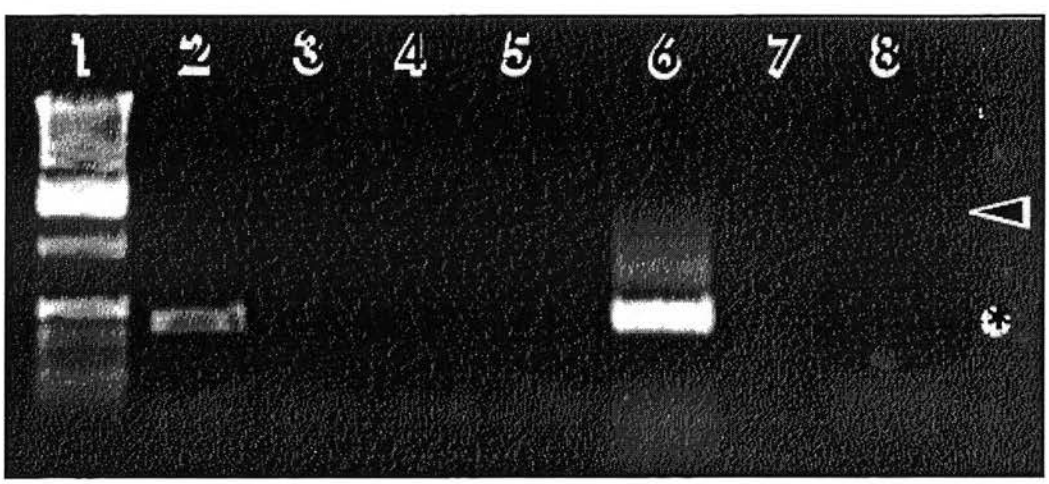
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7.12. *In situ* hybridisation results

No target sequence detection was seen in positive control material under any of the experimental conditions used. All of the tissue sections were completely clear at the end of the procedure. Because of the complete lack of signal, it is not possible to comment on the effect of varying the experimental parameters.

Discussion

Nie-Lin *et al* (1996, 1997) used DNA *in situ* hybridisation for the detection of *Anaplasma* rickettsiae in tick sections. Because rickettsiae are so small (smaller than some species of bacteria), nucleic acid is likely to be present in most histological sections through the organisms, making them highly suitable for detection by DNA *in situ* hybridisation. The zygote and kinete forms of *T. parva* are considerably larger (see Chapter 5) than rickettsial organisms and have a relatively small nucleus. Consequently, there is a high probability that the nucleus will not be present in a section that includes other parts of the cell which may lead to false-negative results. Kinetes are about 19μm long with an anteriorly placed nucleus of 2μm diameter (although mature kinetes can become multinucleate). Unless a kinete was sectioned directly through this region, probe annealing would not occur.

The fluorescein labelling of the TPR1 sequence varied from very poor to non-existant. Discussion with staff at Pharmacia Biotech revealed that some templates are better at labelling than others and the TPR1 sequence may have been unsuitable for an unknown reason. The sequence is not unusual in any way, in terms of nucleotide sequence motifs, so its lack of suitability for fluorescein labelling using the methods described is difficult to understand. The Fl-dCTP was very expensive so a decision was made at this point to concentrate on other labelling options.

Discussion with staff at Vector Laboratories revealed that the thermocoupling process during Biotin labelling had probably renatured the amplified product DNA in such a way that it had formed an enormous molecule that would have been unable to pass through the agarose gel matrix. They recommended using the U.V light

coupling, even though the thermocoupling technique is suggested on their product protocol sheets.

The Biotin labelled TPR1 was dot blotted to confirm that the bands seen in the loading wells were actually the labelled DNA. Going through this procedure was an incremental way of discovering what went wrong at a particular stage of the process.

The gel result demonstrated that the probe was the correct size (slightly bigger than native amplicon because of label incorporation). The membrane detection result demonstrated that the amplicon had been labelled. Finally, the detection of DNA using a Dot Blot demonstrated that the label had been correctly incorporated and that the probe was capable of binding to a complementary sequence.

Photocoupling was more successful than the thermocoupling in that it did not alter the size of the amplicon. Although the probe did incorporate label, it was unable to bind complementary sequences on a membrane. One possibility to explain the result is that the biotin may have been obscuring some of the binding sites. The use of biotin was discontinued after these results were repeatedly obtained as DIG appeared to be a better label.

DIG was more successful than the other two labels in that it consistently labelled the DNA in the PCR, showed a high level of incorporation in the probes and the probes successfully detected complementary DNA on membranes.

No signal was detected in any of the *in situ* hybridisation experiments. There are a number of possibilities which may have contributed to this situation.

It has been shown that DNA is lost from sections during fixation (Hopwood 1982) and during *in situ* hybridisation (Raap *et al* 1986). PCR and membrane detection results give a strong indication that the tissue had suffered a substantial reduction in detectable DNA, whether through loss or damage. The strength of signal produced

from amplifying DNA from the large number of glands was much reduced over what would have been expected from unfixed material. Amplicon yields from some of the ticks in Chapter 3 shows strong bands from only one tick. The salivary glands from the ticks used in this experiment were highly infected and would have produced very intense bands on amplification. The barely discernible amplicon produced from the small bundle of glands represents amplification of DNA from 10 to 20 highly infected salivary glands. The lack of detectable amplification from the sections may be a reflection of the sensitivity of PCR and Dot Blot. The extended fixation times used in this study resulted in loss of, or damage to the DNA and was likely to be a primary reason for the failure of the technique.

Karnovsky's Fixative is a very well established fixative and has been particularly useful in studies where accurate preservation of morphology and chemical activity was necessary. One of the theoretical benefits of the fixative for a study of this nature is that the crosslinking produced by the mixture of the fixatives (formaldehyde and gluteraldehyde) is less than that of the more active of the pair. The material intended for use with the procedure were the ticks from the same experiment described in Chapter 5. Half of the tick batch was embedded and sectioned in methacrylate resin for light microscopy and the other half was wax embedded for the purposes of *in situ* hybridisation. Fixation procedures should be optimised like the rest of the reaction conditions, but under the circumstances, this was not possible. The salivary gland tissue seemed to be unaffected by conditions of Proteinase K digestion many magnitudes above those which other authors have described as extremely detrimental for their tissue samples, suggesting that the proteins were excessively cross-linked.

Different Proteinase K concentrations

No signal was observed with any of the experiments performed, so identifying specific reaction condition variants was not possible. No obvious effect was noted from trypsin treatment with different digestion times and no consistent differences were seen in the morphology of the glands with a range of Proteinase K concentrations up to 300µg/ml. A commonly used Proteinase K concentration for *in*

situ protocols is around 10μg/ml, so it was very surprising not to notice any significant morphological effects at the levels mentioned. The application of 25mg/ml Proteinase K (stock concentration) should have completely digested all of the protein within the section. The morphology of the tissue sections certainly showed signs of proteolytic digestion (Fig. 7.2a and 7.2b), but did not look badly affected. The full length TPR1 amplicon, heat fractionated TPR1, TPR1 and tick sequence oligonucleotide probes all failed to show signal detection. It is possible that the extent of protein cross-linking, and subsequent conformational alteration was so extreme that the enzymes were unable to recognise active binding sites with which they could operate.

The optimum temperature for PK activity is 55^0C, which is the temperature usually used in solution based DNA extraction techniques where efficient protein digestion is required. Efficient protein digestion would be extremely detrimental for *in situ* procedures as the morphology of the tissue sections would be destroyed. The required effect is to change the proteins (which will have been altered from their native state during fixation) surrounding the DNA just enough to allow probe access. A temperature of 37^0C is usually employed for PK digestion steps with concentrations around 5 to 30μl/ml. Nie-Lin *et al* (1996, 1997) used a 42^0C PK step with *D. andersoni* tick sections and that temperature was adopted as a standard in these experiments.

Probe sizes and concentration

The TPR1 amplicon is a reasonable size of probe at 405bp for the purposes of *in situ* hybridisation. However, because of the initial negative results, it was felt that decreasing the size would be a sensible step to take. Heat fractionation of the TPR1 probe was considered to be a substantially quicker and easier method of reducing probe length than restriction enzyme digests. When this proved unsuccessful, the decision to label the TPR1 primers seemed the next logical step. The fact that the primers did not detect the target cells either, indicated that probe access may have been very restricted or the target was highly degraded in some way.

PCR and dot blot detection of DNA within fixed material

PCR and dot blot results suggested that a considerable amount of target sequence degradation had occurred. Hopwood (1982) reported that 30% of the nucleic acid in tissue could be lost through the process of fixation. PCR product yield from the large number of glands was considerably smaller than would have been expected from unfixed material. The formaldehyde concentration may have been too high to allow efficient amplification, as failure to amplify infected ticks preserved in formaldehyde was demonstrated in Chapter 3. The fact that DNA could not be detected from a collection of ten to fifteen highly infected salivary glands (from a tick batch known to be highly infected from microscopical examination) shows there was a very substantial loss of parasite DNA from the samples. DNA from unfixed, single salivary glands was amplified in Chapter 3 and produced powerful amplicons, showing the extent to which the DNA must have been affected. However, as Fig. 7.11 shows, there was a substantial amount of target material available, and it is unlikely that all of the DNA was damaged from all of the areas on the section. Single copy genes have been detected by *in situ* hybridisation within virus infected tissue and there must have been at least one *T. parva* genome sequence intact as the strong PCR band from DNA extracted from the large collection of salivary glands shows. This shows that although it seems likely that DNA loss from the sections could have been responsible for reduced signal intensity, it unlikely to have caused total loss of signal, thereby implicating other factors.

Prehybridisation, stringent hybridisation or washing steps were considered unnecessary. All of these steps are ways to reduce the background, but to use them effectively, a signal must be present. No signal was generated in the sections in any of the experiments making procedures to reduce extraneous signal unnecessary.

In situ hybridisation has been carried out on archival specimens on several occasions (Kangwanpong *et al* 1995, Unger *et al* 1998). Many of these samples are likely to have been over fixed, showing that it may be possible to develop the technique to overcome this problem.

Future work to develop *in situ* hybridisation protocols

Future work with Karnovsky's fixed material may benefit by increasing the temperature of PK digestion above 42^{0}C. Microwave treatment of tissue sections has been reported as part of *in situ* procedures (Leong and Sormunen 1998, Kaneko *et al* 1999) and could be attempted with this material. Initially, removing the Triton X-100 from the post hybridisation wash and making all of the post hybridisation steps as low stringency as possible may allow a signal to be generated. Most factors that can be adjusted to improve the reaction can only be assessed when signal improvement is the aim as opposed to signal generation.

The best way to proceed with developing the technique would be to assess the fixation conditions. Using the tick as target material would be the best way to approach the situation. Unfed ticks could be dissected and placed in different fixatives (10% paraformaldehyde being a good starting point) for varying lengths of time. A range of DIG labelled tick sequence probes could then be applied and assessed for signal strength. The binding of these probes would confirm the optimum fixation conditions for probe size, penetration and signal detection. Once an efficient and specific system had been established, engorged ticks (on uninfected blood) could then be used at different stages throughout the moult to see if the bloodmeal affected the fixation conditions and probe annealing. Once these conditions had been met, the next step would then be to use *T. parva* specific probes on optimally fixed, infected ticks.

CHAPTER 8

General Discussion

New chemotherapeutic agents and acaricides are constantly being produced in the fight against East Coast fever, spurred on by the constant development of parasite and tick resistance to those agents. Disease management is increasingly being seen as the best means of control, and as such, is putting much more emphasis on understanding tick biology and the nature of the tick-*Theileria* relationship. A better understanding of the dynamics of tick infections will be fundamental if management strategies are to be tailored to individual areas and regions with diverse *T. parva* epidemiologies. It is hoped the work presented in this thesis should go some way in preparing the ground for this understanding.

8.1. *PCR as a diagnostic tool*

It is likely that PCR will be used with increasing frequency to analyse field-collected samples for *T. parva* infection. Single primer set reactions provide information on an organism based on hybridisation with only one region of the genome. If the region was invariant throughout a species, the information obtained from PCR analysis would be an accurate reflection of nature. However, if genetic variation in some uncharacterised field strains of the same species did occur in this region of the genome, so that primers were unable to anneal, false negatives could be obtained. Multiple primer set PCR (multiplex PCR) may be the best way to minimise the probability of false negative results. Using two or more primer sets in the amplification reactions, preferably for hybridising to genes on different chromosomes, would mean each *T. parva* sample would be confirmed by two or more amplicons. PCR results from the field samples in Chapter 4 demonstrated an ambiguity that may have been avoided if multiple primer sets had been used. Samples from Kitale and Kakamega produced a 3.2Kb band on amplification with the TPR1 primers. This band size had not been seen from amplification reactions with any of the *T. parva* or other *Theileria* species previously tried. It was not

possible to conclude if the parasites (piroplasms were seen in blood smears) were an uncharacterised *T. parva* or a separate theilerial species. If another primer set had been included in the reaction (i.e. *T. parva* ribosomal gene primers) it may have clarified the situation.

A danger associated with over reliance on PCR as a diagnostic tool is that the specialised skills used for conventional diagnostic techniques may be neglected. Those who know very little about ECf aetiology can carry out PCR analysis very effectively. The ability to diagnose *T. parva* from schizont, piroplasm and clinical signs will always be one of the most important tools in any epidemiological study. PCR analysis allows for high throughput, rapid results, but is best regarded as a complement to the traditional techniques practised by experts in their field.

8.2. *Vaccination*

One of the most difficult aspects of large scale, live sporozoite vaccine production will be standardisation between vaccine batches. Continuing research into tick factors that affect parasite development will be essential if improvements are to be made in this area. However, even if tick infections were successfully manipulated, the use of live, virulent organisms as part of a vaccine regime is inherently risky. Post-vaccination monitoring would almost certainly be required and would increase vaccine costs. Creation of carrier status in vaccinated animals will be a fundamental part of creating or maintaining endemic stability within an area. Insufficient research has been carried out on *T. parva* transmission by *R. appendiculatus* from carrier-state infections to date. The vaccine will require regular boosting by challenge from infected ticks to maintain efficient protection. Investigating the prevalence of tick infections from carrier-state cattle, particularly exotic breeds (as carrier-state transmission in exotic breeds may differ from that in indigenous breeds) will be essential for appropriate tick control strategies to be formulated.

A recombinant antigen vaccine called P67 (Musoke *et al* 1992) is under development at the International Livestock Research Institute, Kenya. Studies have shown that

while animals immunised with the infection and treatment vaccine remain protected for over 3.5 years (Burridge *et al* 1972) after inoculation, the same may not be true for those immunised with the recombinant vaccine (McKeever and Morrison 1998). Antibody titres to P67 were found to decline rapidly after inoculation. However, ongoing work is likely to improve on present weaknesses. The greatest concern for the use of recombinant vaccines is the effect they will have on endemic stability. Endemic stability would be expected to deteriorate as sporozoite inoculation from tick challenge would not be expected to boost immunity (McKeever and Morrison 1998). Because recombinant vaccines will have to be delivered more frequently than infection and treatment vaccines, they may be more appropriate for use in farms where extensive management procedures are adopted to reduce tick transmission of *T. parva*. These farms are more likely to stock Grade cattle and rely less on endemic stability as a means of reducing stock loss than less intensively managed farms, such as those run by small holder dairy farmers. In the case of the latter, a live vaccine may be more appropriate, as a recombinant vaccine induced breakdown of endemic stability could lead to increased incidence of disease.

T. parva Lanet is a mild strain of *T. parva* that has shown promise as a live vaccine candidate (Mbogo *et al* 1996). The immunised cattle were solidly immune against challenge from virulent *T. parva* isolates and the clinical reactions to inoculation were so mild that administration of tetracyclines were unnecessary. *T. parva* Icely showed the same promise when it was isolated in the 1960's, but rapidly became very virulent after continual tick-cattle passage (Barnett and Brocklesby 1961, Brocklesby and Bailey 1968). It is possible that under the same circumstances, *T. parva* Lanet would behave in the same way as it became more adapted to the transmission system. Tick sporozoite stabilate was not produced in the 1960s which meant cattle infection required application of infected ticks - thereby necessitating continual transmissions to supply the ticks. Today, large quantities of *T. parva* Lanet bulk stabilate could be made, eliminating the need for continual passage. This would obviously not represent a permanent solution as the vaccine stocks would be limited and replacement would require further passaging and risk the development of

increasing virulence. It may, however, be a worthwhile solution for delivering a cheap, safe (relative to the present infection and treatment *T. parva* stocks) and effective (relative to P67) vaccine in the immediate future. This would allow time to develop further the recombinant and infection and treatment vaccines.

8.3. T. parva *transmission from carrier-state infections*

Research into transmission dynamics from carrier-state infections is likely to become an increasingly important field. Areas in which live, sporozoite vaccines are delivered are likely to see an increase in the number of *T. parva* carrier cattle within them. If tick transmission dynamics from carrier-state infections are to receive further study, laboratory data may prove more revealing than data from the field. Results from Chapter 5 showed that high infection abundance can be produced in ticks from very low piroplasm parasitaemias, representative of carrier-state infections. Animals in endemically stable areas are likely to harbour carrier-state infections and field collected *R. appendiculatus* characteristically have very low *T. parva* abundance. The laboratory and field results seem to be somewhat at odds with each other, as one suggests high tick infections can occur with carrier-state infections and the other suggest they do not. *T. parva* associated tick pathology, leading to high mortality was demonstrated in Chapter 6. Field ticks, collected and analysed to obtain salivary gland infection data may represent the survivors of a larger population that were exposed to *T. parva*. Those with high parasite load may have been rendered less fit and died, which may allow for a skewed interpretation of the infection data.

8.4. *Dynamics of* T. parva *in* R. appendiculatus

Examination of salivary glands from field and/or laboratory strains of *R. appendiculatus* exposed to *T. parva* infection always reveals an overdispersed distribution. The quantitative results from the histological sections in Chapter 5 suggest that this overdispersion is apparent in all *T. parva* forms, from sexual stages to sporoblasts, throughout the tick moult. With this in mind, the results from the experiment that compared the adult infections in the two batches of ticks with different *T. parva* infection histories (one of which had been exposed to *T. parva* as

larvae and nymphs, the other only as nymphs) were very surprising. The vast majority of twice infected ticks contained no infection, and those that were infected, harboured very few sporoblasts. The fact that such a high proportion of ticks were uninfected (81% compared with 32% in the once infected controls), suggested a fundamental immune mechanism had been activated as a consequence of previous exposure to *T. parva*. Since this mechanism appeared so universal amongst the tick batch investigated, it may represent a worthy target for future investigation of *T. parva* control methods. Sporoblasts were not detected in the salivary glands of the nymphs after larval exposure to *T. parva*. This suggests an acquired immune response developed in the nymph-adult ticks based on primary infection in the gut and/or haemocoel. The *T. parva* forms that appear most vulnerable to potential tick immunity are the zygote and kinete. During the moult, zygotes remain in the gut digestive cells for around two weeks and are relatively static until they mature rapidly prior to transformation to the kinete form. The parasite development may proceed in this manner in order to reduce the metabolic demands on the host during the moulting process, or the mature zygote or kinete may be vulnerable to cytoplasmic destruction as demonstrated with *Plasmodium* parasites (Vernick *et al* 1995). The gut and haemocoel may be the best tick compartments on which to focus initial research for a transmission blocking vaccine approach in order to target the zygote and kinete forms. Direct kinete penetration into salivary glands has never been shown by microscopy that may suggest that acinar penetration is very rapid. No kinetes were seen in the haemolymph of any ticks examined in the course of the experiments described here. Phenoloxidase-like material and gut derived products were seen on the salivary glands of adult ticks that were infected with *T. parva* as larvae and nymphs (see Chapter 5). The affected areas were specifically where the salivary glands and gut were in direct contact, suggesting that kinetes may penetrate salivary gland acini from juxtaposed gut tissue. This would minimise parasite contact with the haemolymph, which may represent a hostile environment for the parasite.

Empirically, parasite distributions are often best described by the negative binomial distribution; some hosts have many parasites while most have just a few (Wilson *et*

al 1996). This phenomena is seen throughout a wide spectrum of parasitic infections from helminths in African lions (MullerGraf *et al* 1999) and freshwater fish (Baling and Pfeiffer 1997) to ectoparasites on birds (Lee and Clayton 1995, Heeb *et al* 1996). Overdistribution has also been seen in field infections of *Theileria* in *Hyalomma detritum* (Flach *et al* 1993) and *Babesia* in *Boophilus microplus* (Guglielmone *et al* 1997). The results from laboratory tick infections reported in this thesis show *T. parva* in *R. appendiculatus* follows this pattern as has been demonstrated previously (Büscher and Otim 1986). *T. parva* sporoblast infections in *R. appendiculatus* develop after a multitude of complex parasite-tick interactions at every stage throughout the tick moult. Numerous individual variations in tick and parasite populations will impact on every aspect of this relationship and lead to a multitude of possible infection outcomes. These variations are likely to include; age of piroplasms during infection, host blood factors which may change over the course of infection and during tick feeding, novel parasite recombination events in the tick gut, tick bloodmeal volume, pH of gut contents, efficiency and speed of bloodmeal digestion, agglutinin (lectin) concentration, haemocyte quantity, presence of immune factors such as antibacterial peptides, size of salivary glands etc. *Theileria* refractory tick strains are likely to have been naturally selected over susceptible strains. The majority of ticks will tend greatly to reduce parasite survival, but a small proportion of ticks will vary sufficiently from the majority in one or more of the above factors, to allow increased parasite survival. A smaller proportion still, will vary to such an extent that very large numbers of parasites will survive.

8.5. *Tick pathology and potential immunity*

A large amount of variation in sporoblast maturity and salivary gland pathology was evident within individual glands. The developing sporoblast causes massive hypertrophy of the host cell (e-cells in Type III acini) nucleus and a substantial alteration of the cell metabolism (Fawcett *et al* 1982). Glycogen levels dramatically increase in the infected cell cytoplasm and the amount of secretory products are greatly reduced. Binnington (1980) suggested that 'the parasite [*Theileria parva*] may rely upon a switching on of protein synthesis in the [salivary] gland by feeding'

to allow the enormous growth in its own structure up to sporozoite formation. Spontaneous degeneration of d- and e-cells in the glands of uninfected ticks is commonly seen in the salivary glands of uninfected ticks in the late stages of feeding. Fawcett *et al* (1982) suggested that their degeneration was as a consequence of their role in the feeding process being completed. It was then argued that *T. parva* actually extended the life of e-cells it parasitised, because the cells are maintained until sporozoite elimination, a period which may be much extended over the lifetime of some unparasitised cells. Parallels can be drawn between the mechanisms by which *T. parva* sporoblasts alter the acinar host cell metabolism and *Trichinella spiralis* effects control over mammalian muscle cells. *T. spiralis* is an intracellular nematode that can form a Nurse cell complex in mammalian striated skeletal muscle cells (Purkerson and Despommier 1975). The parasite is enclosed within a collagen capsule (Teppema *et al* 1973) and induces transformation of the muscle tissue to recruit nutrients to, and export metabolic waste (Stewart 1983) from, the encapsulated parasite. A *T. spiralis* secreted antigen was found to localise to the cytoplasm and nuclei of infected cells. It was believed to be involved in maintaining the redifferentiated state of the infected muscle cells throughout the life of the Nurse cell complex (Despommier *et al* 1990). The occurrence of foreign proteins within the nuclei of infected host cells has been documented for viruses (Kops and Knipe 1988). Adenoviruses produce proteins which interfere with host cell DNA and protein synthesis (Babiss and Ginsberg 1984).

One of the questions that arose from the work on parasite induced, tick pathology in Chapter 6 was why some acini were pathologically affected, while others supported parasite development without any obvious ill effects. One possibility is that parasites that established ineffective control over the host cell nuclei may have initiated a cell death response in the acinar cell that ensured the parasite would also be destroyed.

8.6. *Transgenic ticks*

Interest in transgenic ticks refractory to *T. parva* infection is likely to become an area of increased interest in the future. If complete eradication of the disease was desired,

ticks would have to be rendered completely refractory to infection. However, if endemic stability was the desired result in an area of transgenic tick introduction, complete refractoriness would be inappropriate. The ideal aim in this situation may be high prevalence, low abundance infections in male ticks only. Because male ticks often tend to interrupt their feeding to mate and sporoblasts are smaller in male ticks (compared to females), they tend to deliver smaller sporozoite quantities over longer periods than female ticks. This would allow cattle to develop protective immune responses without being overwhelmed by challenge. These issues are academically interesting, but are unlikely to ever become a reality. It is difficult to imagine how a genetically modified line of ticks could outcompete and replace field ticks. The vast majority of field *R. appendiculatus* carry no *T. parva* infection (Walker *et al* 1981) and therefore could not be regarded as 'less fit' than their transgenic counterparts.

8.7. *Future work on parasite-tick interactions*

Future work to develop *in situ* hybridisation for the detection of *T. parva* in *R. appendiculatus* would be very valuable. Although antibody based techniques may have a theoretical advantage over nucleic acid based approaches, a great deal of work would have to be completed first on *in vitro* culture methods to obtain different parasite lifecycle forms relevant to the tick, or more efficient purification techniques to obtain them directly from infected, moulting ticks. This would allow common antigens to be investigated or polyclonal sera to be raised against all tick forms. Monoclonal antibodies would probably be too specific for this purpose. A monoclonal antibody that was highly specific for a zygote, may not recognise a kinete and potentially give misleading information. Would it recognise a zygote when it was transforming into a kinete? At what point would the parasite no longer be a zygote, and when could it be considered a kinete?

Isolating zygote and kinete forms would make the study of tissue invasion and survival mechanisms considerably easier. This could have important consequences for a transmission blocking vaccine approach. Developing better artificial feeding systems than are presently available for ticks would allow very significant progress

to be made in this area. A standardised concentration of parasites in the blood medium, could be used throughout a transmission experiment and vastly increase the potential for controlling parasite intake and manipulating the infection outcome.

8.8. *Summary*

This thesis has shown that *T. parva* infection in *R. appendiculatus* causes pathology in a proportion of the tick population. These pathologies are characterised by perforated guts, which cause leakage of gut contents into the haemocoel, perforated and malformed Malphighian tubules, damaged salivary glands acini and malformed or missing legs, possibly as a consequence of secondary fungal infection. There is a possibility that *T. parva* is vulnerable to *R. appendiculatus* immune mechanisms. The parasite lifecycle has been examined throughout the period of the tick moult, and the results indicate that zygote and kinete forms appear most susceptible to reductive mechanisms. The phenoloxidase cascade is likely to play a role in this process and may be affected in the haemolymph.

Very little, if any, research attention has been focused on tick pathology in relation to ECf. However, it is possible that the phenomena described in this thesis could have significant influences on *T. parva* and *R. appendiculatus* population dynamics in ECf endemic areas. Further work in this area, particularly on tick transmission from carrier-state infections could make significant contributions to our understanding of, and our ability to successfully manipulate the disease in the field.

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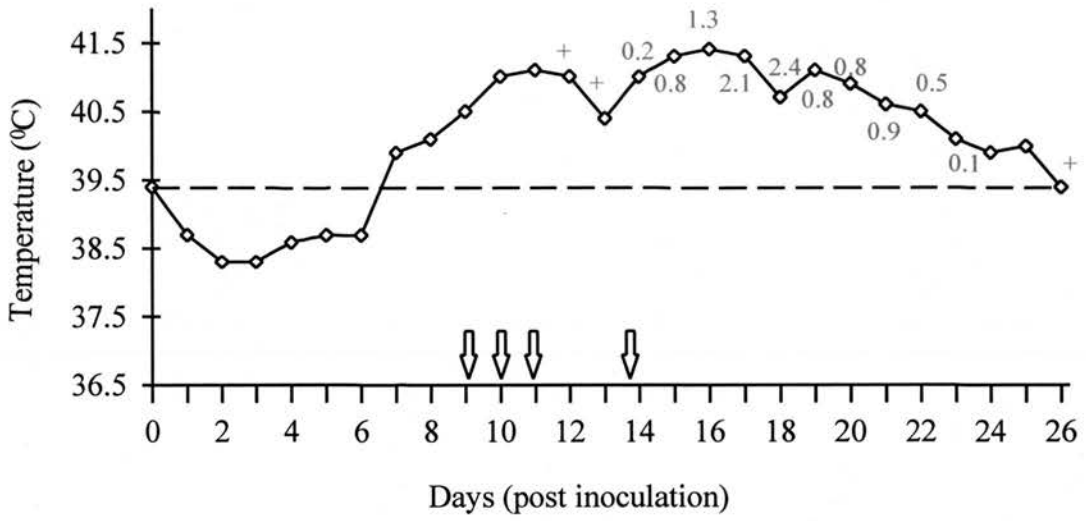
APPENDIX 1

The data included in this section relates to experiments carried out in Chapter 3.



Calf 552. 0.1ml *T. parva* Muguga Stabilate 71 (0.25t.e - 30 sporoblasts). ⇩ - Tick application. The red figures represent the piroplasm parasitaemia on those days. Temperatures exceeding 39.5°C (represented by the dashed line) are considered a pyrexia. + represents piroplasm parasitaemias of less than 0.1% infected erythrocytes.

Table 1A. (overleaf) contains count data from MGP stained, paired salivary glands. One of the aims of the experiment was to compare MGP and PCR infection data from salivary glands from individual ticks. It was therefore essential to first confirm that the quanta of infection in each gland was highly correlated using just MGP.

Table 1B. (overleaf) contains MGP and PCR data for individual ticks chosen from 3 groups of ticks to show the correlation between the two techniques and whether the PCR could be employed to measure the prevalence and abundance of tick infections. The PCRs measurement of abundance would be on a 0 to 5 scale as opposed to exact values obtained from microscopy.

Table 1A. *T. parva* detection in MGP stained paired salivary glands from 7 groups of adult ticks which fed as nymphs on calf 552. % Piros: percentage piroplasm parasitaemia. m / f: male / female tick. ↑: increasing parasitaemia. ↓: decreasing parasitaemia.

% Piros	m / f	Left	Right	% Piros	m/f	Left	Right
↑ + - +	m	0	0	↓ 2.4 - 0.8	m	1	1
	m	1	0		m	29	34
	m	0	0		m	77	58
	m	0	0		m	11	36
	m	0	0		m	66	71
	f	0	0		f	0	0
	f	0	0		f	72	107
	f	0	0		f	61	62
	f	0	0		f	94	93
	f	0	0		f	1	1
↑ + - 0.2	m	4	0	↓ 0.5 - 0.1	m	0	0
	m	0	0		m	2	4
	m	56	62		m	1	1
	m	0	0		m	2	0
	m	15	6		m	23	12
	f	0	1		f	0	0
	f	9	2		f	0	0
	f	7	3		f	0	0
	f	0	0		f	0	4
	f	0	0		f	59	58
↑ 0.2 - 0.8	m	1	1	↓ + - +	m	0	0
	m	2	3		m	0	0
	m	179	201		m	0	0
	m	203	201		m	0	0
	m	38	52		m	0	0
	f	182	154		f	0	0
	f	149	118		f	0	0
	f	54	53		f	0	0
	f	197	200		f	0	0
	f	65	43		f	0	0
↑ 1.3 - 2.1	m	154	142				
	m	254	268				
	m	147	117				
	m	1	0				
	m	9	10				
	f	130	129				
	f	283	291				
	f	97	100				
	f	15	8				
	f	206	170				

Table 1B. PCR and MGP were compared with 3 groups of 30 ticks (15 males / 15 females). PCR values were ranked on a scale of 0 - 5 which represented an relative grading of amplicon intensity (0 being no amplicon and no infection and increasing to 5 which were very intense amplicons representing a large infection). The figures in the MGP columns represent actual sporoblast count data. % Piros: percentage piroplasm parasitaemia. m / f: male / female tick. ↑: increasing parasitaemia. ↓: decreasing parasitaemia.

% Piros	m / f	PCR	MGP	% Piros	m / f	PCR	MGP
↑ + - +	m	1	-	↑ + - 0.2	m	4	-
	m	-	-		m	1	-
	m	-	1		m	4	-
	m	-	-		m	4	9
	m	-	-		m	-	-
	m	2	1		m	4	7
	m	1	1		m	-	-
	m	-	-		m	4	33
	m	1	-		m	4	81
	m	-	-		m	4	14
	m	-	-		m	1	-
	m	-	-		m	1	-
	m	-	-		m	3	2
	m	2	1		m	2	2
	m	2	3		m	-	-
	f	-	2		f	4	3
	f	-	-		f	3	-
	f	-	-		f	1	-
	f	-	-		f	3	8
	f	-	-		f	3	12
	f	-	-		f	3	19
	f	-	-		f	-	-
	f	-	-		f	-	-
	f	1	-		f	3	14
	f	-	-		f	1	1
	f	-	-		f	4	57
	f	-	1		f	2	4
	f	-	-		f	3	19
	f	3	1		f	4	38
	f	-	-		f	3	5

Table 1B. (continued)

% Piros	m / f	PCR	MGP	% Piros	m / f	PCR	MGP
↓2.4 - 0.8	m	5	86	↓2.4 - 0.8	f	5	66
	m	5	91		f	4	3
	m	5	46		f	4	15
	m	2	1		f	1	5
	m	3	6		f	3	4
	m	5	138		f	-	2
	m	4	20		f	5	76
	m	5	31		f	3	3
	m	5	28		f	4	28
	m	2	14		f	4	115
	m	5	43		f	1	12
	m	5	196		f	3	22
	m	5	5		f	3	97
	m	5	10		f	1	7
	m	-	3		f	1	8

Table 1C. The sporoblast count data shown is shown alongside the PCR ranked values from the paired salivary gland. This table shows the same data as Table 3B, but in a form that allows easier comparison of the MGP and PCR data.

PCR RANKED VALUE					
0	1	2	3	4	5
0 (x 54)	0 (x 7)	1	0	0	5
1	1	1	1	0	10
1	1	1	2	3	28
2	5	2	3	3	31
2	7	3	4	7	43
3	8	4	5	9	46
	12	14	6	14	66
			8	15	76
			12	20	86
			14	28	91
			19	33	138
			19	38	196
			22	57	
			97	81	
				115	
Mean Number of Sporoblasts					
0.15	2.62	3.71	15.14	28.20	68.00

Table 1D. PCR was used on fed and unfed adult ticks from two batches which detached at piroplasm parasitaemias of 0.2% and 2.1%. Fifteen ticks from each group were tested, and the PCR ranked values are displayed for each. * m: male tick, f: female tick, fed: the ticks were partially fed on a rabbit for 4 days.

0.2% Piroplasm Parasitaemia				2.1% Piroplasm Parasitaemia			
m unfed*	m fed	f unfed	f fed	m unfed	m fed	f unfed	f fed
3	4	1	5	1	5	2	5
-	4	3	1	3	5	4	5
4	5	3	1	2	5	2	5
-	5	1	4	2	5	3	5
3	5	-	1	3	5	3	4
1	1	-	1	3	5	2	4
1	5	-	3	3	1	3	4
-	1	2	4	2	5	3	3
1	5	3	1	3	5	4	3
2	5	2	2	2	5	3	4
-	4	3	2	1	5	2	3
3	5	-	3	3	5	3	3
1	1	-	3	1	5	3	3
3	5	1	1	3	5	2	4
-	4	-	4	2	5	2	2
Number Infected / Total Number Tested							
10 / 15	15 / 15	9 / 15	15 / 15	15 / 15	15 / 15	15 / 15	15 / 15
Mean PCR Ranked Value							
1.47	3.73	1.27	2.40	2.27	4.73	2.73	3.8

APPENDIX 2

The data included in this section relates to experiments carried out in Chapter 4.

Table 2A. (overleaf). The numbers and species of tick samples collected from each animal in the three field sites are displayed, as well as the PCR and smear examination results from the blood samples taken from those animals. * - the two values displayed in the piroplasm detection column represent the PCR result / bloodsmear result. The PCR results refer to detection of *Theileria* species only (further details are given in Table 2E).

Tables 2B, 2C and 2D. (overleaf). All the tick samples processed from Kitale, Kakamega and Limuru are displayed in the tables. Blood samples are included as well, but only from cattle that had ticks removed from them (i.e. blood samples taken from tick-free cattle are not displayed in these tables). The results from the blood samples are shown alongside the tick results because they help clarify the interpretation of results obtained from dead ticks that could not be dissected.

Explanation of table abbreviations. TPR1: *T. parva* specific primers used. 989 / 990: *Theileria* sp. primers used. GroE: *C. ruminantium* specific primers used. Piro (%): piroplasm parasitaemia obtained from bloodsmear examination. RA: *Rhipicephalus appendiculatus*. AM: *Amblyomma variegatum*. BO: *Boophilus decoloratus*. m: male tick. f: female tick. n: nymph. (sg): salivary glands. (d): dead tick, therefore dissection was not possible. These ticks were bisected and DNA was extracted from the whole body. + (3.2Kb): 3.2Kb amplicon obtained from amplification with the TPR1 primers, as opposed to the expected size of 402 - 405bp. n.d: not done.

Table 2E. All of the blood sample results as assessed by the PCR and smear examination from the three sites are shown.

Table 2A. All of the animals sampled from the field sites are recorded along with the number and species of tick collected from them. *R. app*: *R. appendiculatus*. *R. eve*: *R. evertsi*. *B. dec*: *Boophilus decoloratus*. *A. var*: *A. variegatum*.

	No. Ticks Collected						
Collection Area	Sample No.	Animal Code	Piros*	*R. app* m / f	*R. eve* m / f	*B. dec* m / f	*A. var* m / f
Limuru	37	GAT 2/X	- / <0.1	4/0	1/0		
		GAT 4/2	n.d	5/2			
		GAT 6/3	- / -				
		GAT 6/4	- / <0.1				
		GAT 8/X	- / -				
		GAT 15/X	- / -				
		GAT 15/X2	- / -	0/1			
		JET 2/X	- / -	4/1			
		JET 2/6	+ / <0.1	2/0			
		JET 7/1	- / -	0/2			
		JET 18/1	- / -				
		JET 18/3	+ / -				
		JET 18/7	- / -			0/1	
		THI 21/X	- / -	2/2			
		THI 36/2	- / <0.1	6/17,2n	3/0	0/6,1n	
		THI 36/4	+ / <0.1	16/10	1/0	0/6	
		THI 42/4	- / n.d	98/12,7n	4/2,2n	3/21,1n	
		THI 42/6	+ / <0.1	25/9	4/1	3/11	
		THI 58/1	- / -	36/15,10n	2/1,1n	1/7	
		THI 58/X	- / -	9/15,1n			
		THI 58/X2	+ / <0.1	25/15		10/6	
		KANY 8/2	- / -	1/1			
		KANY 8/X	- / -	1/1			
		KANY 8/X2	- / -	2/0			
		KANY 9/1	- / <0.1	2/10	2/0		
		KANY 9/X	+ / -	12/10			
		KANY 10/2	- / -	9/6	3/0		
		KANY 11/1	+ / 0.2	2/3			
		KANY 11/4	- / +	2/1			
		KANY 11/5	+ / 0.2	3/2			
		KANY 11/X	- / -	4/1			
		KANY 11/X2	+ / -	4/1			
		NDA 24	- / -	61/16			
		107	- / n.d				
		185	- / n.d				
		175	+ / n.d				
		SHEEP	n.d		1/7		
Kitale	7	1.88	+ / 0.2			0/12	
		2.82	+ / 0.1				
		F2A1	+ / 0.1	2/1			
		F2A2	+ / 0.3	7/5	2/1	0/3	
		F2A3	+ / 0.1	5/2	2/2	0/3	12/4
		F2A4	+ / 0.3	8/6	2/1	0/3	2n
		F3S	n.d	4/2			

Table 2A. (continued).

				No. Ticks Collected			
Collection Area	Sample No.	Animal Code	Piros	*R. app* m / f	*R. eve* m / f	*B. dec* m / f	*A. var* m / f
Kakamega	19	477	+ / 0.2	6/5			
		481	+ / <0.1	8/1		0/1	2/1
		434	+ / 0.7				
		435	+ / 0.8	16/7		0/1	13/10,4n
		441	+ / 0.1	17/3	1/1	0/5	6/1
		F4A1	+ / <0.1	1/0, 1n			7/3
		F4A2	+ / 0.2	2/2			
		F5A1	+ / 0.2	4/0			11/2
		F5A2	+ / 0.1	3/2		0/1	5/1
		F5A3	+ / <0.1				
		F6A1	+ / 0.5	8/6		0/4	7/3
		F6A2	+ / 0.1				
		F6A3	+ / 0.1				
		F7K	n.d				2/1, 1n
		F8A1	+ / <0.1	2/3		0/1	4/3
		F9A1	+ / <0.1			0/5	1/2
		F10A1	+ / <0.1	9/8, 14n		0/10	25/2, 5n
		F10A2	+ / 0.2				
		F11A1	+ / 0.4	3/4			

Table 2B. The PCR results from blood and tick samples from Kitale are displayed.

KITALE								
	Blood				Ticks			
Animal	TPR1	989 / 990	GroE	Piros (%)	Tick Code	TPR1	989 / 990	GroE
FARM 2								
F2A1	+ (3.2Kb)	+	-	0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	+	-	n.d
					RAf1 (d)	+	-	n.d
F2A2	+ (3.2Kb)	+	-	0.3	RAm1 (sg)	-	+	n.d
					RAm2 (sg)	-	-	n.d
					RAm3 (sg)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (sg)	-	+	n.d
					RAf3 (sg)	-	-	n.d
					REm1 (sg)	-	-	n.d
					REm2 (d)	-	-	n.d
					REf1 (d)	-	-	n.d
F2A3	+ (3.2Kb)	+	-	0.1	RAm1 (d)	-	-	n.d
					RAm2 (sg)	-	-	n.d
					RAm3 (sg)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (sg)	-	-	n.d
					AMm1 (sg)	n.d	-	-
					AMm2 (sg)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMm4 (d)	n.d	-	-
					AMf1 (sg)	n.d	+	-
					AMf2 (sg)	n.d	-	-
					AMf3 (sg)	n.d	+	-
					AMf4 (d)	n.d	-	-
					REm1 (d)	-	-	n.d
					REm2 (sg)	-	-	n.d
					REf1 (sg)	-	-	n.d
					REf2 (d)	-	-	n.d
F2A4	+ (3.2Kb)	+	-	0.3	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (sg)	-	-	n.d
					RAf3 (sg)	+	+	n.d
					AMn1 (sg)	n.d	-	-
					AMn2 (sg)	n.d	-	-
					REm1 (d)	-	-	n.d
					REm2 (d)	-	-	n.d
					REf1 (d)	-	-	n.d
FARM 3								
SHEEP	n.d	n.d	n.d	n.d	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAm4 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d

Table 2C. The PCR results from blood and tick samples from Kakamega are displayed.

KAKAMEGA								
	Blood				Ticks			
Animal	TPR1	989 / 990	GroE	Piros (%)		TPR1	989 / 990	GroE
FARM 1								
477	+ (3.2Kb)	+	-	0.2	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d
					RAf3 (d)	-	-	n.d
481	+ (3.2Kb)	+	-	<0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
FARM 3								
435	+ (3.2Kb)	+	-	0.8	RAm1 (sg)	-	-	n.d
					RAm2 (sg)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAm4 (sg)	-	-	n.d
					RAm5 (d)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (sg)	-	-	n.d
					RAf3 (sg)	-	-	n.d
					RAf4 (sg)	-	-	n.d
					RAf5 (d)	-	-	n.d
					RAf6 (d)	-	-	n.d
					RAf7 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (d)	n.d	-	-
					AMf3 (d)	n.d	-	-
					AMn1 (d)	n.d	-	-
					AMn2 (d)	n.d	-	-
					AMn3 (d)	n.d	-	-
441	+ (3.2Kb)	+	-	0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d
					RAf3 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (d)	n.d	-	-
					REm1 (d)	-	-	n.d
					REf1 (d)	-	-	n.d

Table 2C. (continued).

KAKAMEGA								
	Blood				Ticks			
Animal	TPR1	989 / 990	GroE	Piros (%)		TPR1	989 / 990	GroE
FARM 4								
F4A1	+ (3.2Kb)	+	-	<0.1	AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (sg)	n.d	-	-
					AMf3 (sg)	n.d	-	-
F4A2	+ (3.2Kb)	+	-	0.2	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (sg)	-	-	n.d
FARM 5								
F5A1	+ (3.2Kb)	+	-	0.2	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAm4 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (sg)	n.d	-	-
F5A2	+ (3.2Kb)	+	-	0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
FARM 6								
F6M	+ (3.2Kb)	+	-	0.3	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (sg)	-	-	n.d
					RAf3 (sg)	-	-	n.d
					AMm1 (sg)	n.d	-	-
					AMm2 (sg)	n.d	-	-
					AMm3 (sg)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (d)	n.d	-	-
					AMf3 (d)	n.d	-	-
FARM 7								
F7K	n.d	n.d	n.d	n.d	AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMf1 (d)	n.d	-	-
					AMn1 (sg)	n.d	-	-

Table 2C. (continued).

KAKAMEGA								
	Blood				Ticks			
Animal	TPR1	989 / 990	GroE	Piro (%)		TPR1	989/990	GroE
FARM 8								
F8K	+ (3.2Kb)	+	-	<0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d
					RAf3 (d)	-	-	n.d
					AMm1 (d)	n.d	-	-
					AMm2 (d)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (sg)	n.d	-	-
					AMf3 (sg)	n.d	-	-
FARM 9								
F9K	+ (3.2Kb)	+	-	<0.1	AMm1 (d)	n.d	-	-
					AMf1 (d)	n.d	-	-
					AMf2 (d)	n.d	-	-
FARM 10								
F10K	+ (3.2Kb)	+	-	<0.1	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	-	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (d)	-	-	n.d
					RAf2 (d)	-	-	n.d
					RAf3 (d)	-	-	n.d
					RAn1 (sg)	-	-	n.d
					RAn2 (sg)	-	-	n.d
					RAn3 (d)	-	-	n.d
					AMm1 (sg)	n.d	-	-
					AMm2 (sg)	n.d	-	-
					AMm3 (d)	n.d	-	-
					AMf1 (sg)	n.d	-	-
					AMf2 (d)	n.d	-	-
					AMn1 (sg)	n.d	-	-
					AMn2 (d)	n.d	-	-
					AMn3 (sg)	n.d	-	-
FARM 11								
F11K	+ (3.2Kb)	+	-	0.4	RAm1 (d)	-	-	n.d
					RAm2 (d)	-	+	n.d
					RAm3 (d)	-	-	n.d
					RAf1 (sg)	-	-	n.d
					RAf2 (d)	-	-	n.d
					RAf3 (d)	-	-	n.d

Table 2D. The PCR results from blood and tick samples from Limuru are displayed.

LIMURU											
Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR
KANY 8/2	- / -	RAm1 (sg)	-	KANY 9/X	+ / -	RAf1 (sg)	-	KANY 11/4	+ / -	RAm2 (sg)	-
		RAf1 (sg)	-			RAf2 (sg)	-			RAf1 (sg)	-
						RAf3 (sg)	-				
KANY 8/X	- / -	RAm1 (sg)	-			RAf4 (sg)	-	KANY 11/5	+ / 0.2	RAm1 (sg)	-
		RAf1 (sg)	-			RAf5 (sg)	-			RAm2 (sg)	-
						RAf6 (sg)	-			RAm3 (sg)	-
KANY 8/X2	- / -	RAm1 (sg)	-			RAf7 (sg)	-			RAf1 (sg)	-
		RAm2 (sg)	-			RAf8 (sg)	-			RAf2 (sg)	-
						RAf9 (sg)	-				
KANY 9/1	- / <0.1	RAm1 (dX6)	-			RAf10 (d)	-	KANY 11/X	- / -	RAm1 (sg)	-
		RAm2 (d)	-							RAm2 (sg)	-
		RAm3 (d)	-	KANY 10/2	- / -	RAm1 (sg)	-			RAm3 (sg)	-
		RAm4 (d)	-			RAm2 (sg)	-			RAm4 (sg)	-
		RAf1 (sg)	-			RAm3 (sg)	-			RAf1 (sg)	-
		RAf2 (sg)	-			RAm4 (sg)	-				
		RAf3 (sg)	-			RAm5 (dX5)	-	KANY 11/X2	+ / -	RAm1 (sg)	-
		RAf4 (sg)	-			RAf1 (sg)	-			RAm2 (sg)	-
		RAf5 (dX3)	-			RAf2 (sg)	-			RAm3 (sg)	-
		RAf6 (d)	-			RAf3 (sg)	-			RAm4 (sg)	-
		RAf7 (d)	-			RAf4 (sg)	-			RAf1 (sg)	-
		RAf8 (d)	-			RAf5 (sg)	-				
		REm (dX2)	- / -			RAf6 (sg)	+	THI 21/X	- / -	RAm1 (sg)	-
						REm1 (dX3)	- / -			RAm2 (sg)	-
KANY 9/X	+ / -	RAm1 (sg)	-							RAf1 (sg)	-
		RAm2 (sg)	-	KANY 11/1	+ / 0.2	RAm1 (sg)	-			RAf2 (sg)	-
		RAm3 (sg)	-			RAm2 (sg)	-				
		RAm4 (sg)	-			RAf1 (sg)	-	THI 36/2	- / <0.1	RAm1 (d)	-
		RAm5 (sg)	-			RAf2 (sg)	-			RAm2 (d)	-
		RAm6 (sg)	-			RAf3 (sg)	-			RAm3 (d)	-
		RAm7 (dX3)	-							RAm4 (dX3)	+
		RAm8 (dX3)	-	KANY 11/4	+ / -	RAm1 (sg)	+			RAf1 (sg)	-

Table 2D. (continued).

LIMURU											
Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR
THI 36/2	- / <0.1	RAf2 (sg)	-	THI 36/4	+ / <0.1	RAf2 (dX3)	-	THI 42/4	- / n.d	BOf3 (d)	-
		RAf3 (sg)	-			RAf3 (dX3)	-			BOn1 (d)	-
		RAf4 (sg)	-			RAf4 (d)	+				
		RAf5 (sg)	-			REm1 (d)	- / -	THI 42/6	+ / <0.1	RAm1 (sg)	-
		RAf6 (sg)	-			BOf1 (dX3)	+			RAm2 (sg)	-
		RAf7 (sg)	-			BOf2 (dX2)	+			RAm3 (sg)	-
		RAf8 (sg)	-			BOf3 (sg)	-			RAm4 (d)	-
		RAf9 (sg)	-							RAm5 (d)	-
		RAf10 (sg)	-	THI 42/4	- / n.d	RAm1 (d)	-			RAm6 (d)	-
		RAf11 (sg)	-			RAm2 (d)	-			RAf1 (sg)	-
		RAf12 (sg)	-			RAm3 (d)	-			RAf2 (d)	-
		RAf13 (sg)	-			RAm4 (d)	-			RAf2 (d)	-
		RAf14 (sg)	-			RAm5 (d)	-			RAf3 (d)	-
		RAf15 (d)	-			RAf1 (d)	-			REm1 (d)	-
		RAf16 (d)	-			RAf2 (d)	-			REm2 (d)	-
		RAf17 (d)	-			RAf3 (d)	-			REm3 (d)	-
		RAn1 (dX2)	-			RAn1 (d)	-			REf1 (d)	-
		REm1 (dX3)	- / -			RAn2 (d)	-			BOm1 (d)	-
		BOf1 (d)	-			RAn3 (d)	-			BOm2 (d)	-
		BOf2 (dX3)	-			REm1 (d)	- / -			BOm3 (d)	-
		BOf2 (dX2)	-			REm2 (d)	- / -			BOf1 (d)	-
		BOn1 (d)	-			REm3 (d)	- / -			BOf2 (d)	-
						REf1 (d)	- / -			BOf3 (d)	-
THI 36/4	+ / <0.1	RAm1 (dX4)	-			REf2 (d)	- / -				
		RAm2 (dX4)	-			REn1 (d)	- / -	THI 58/1	- / <0.1	RAm1 (sg)	-
		RAm3 (dX4)	-			REn2 (d)	- / -			RAm2 (sg)	-
		RAm4 (d)	-			BOm1 (d)	-			RAm3 (sg)	-
		RAm5 (d)	-			BOm2 (d)	-			RAm4 (sg)	-
		RAm6 (d)	-			BOm3 (d)	-			RAm5 (sg)	-
		RAm7 (d)	-			BOf1 (d)	-			RAm6 (sg)	-
		RAf1 (dX3)	-			BOf2 (d)	-			RAm7 (sg)	-

Table 2D. (continued).

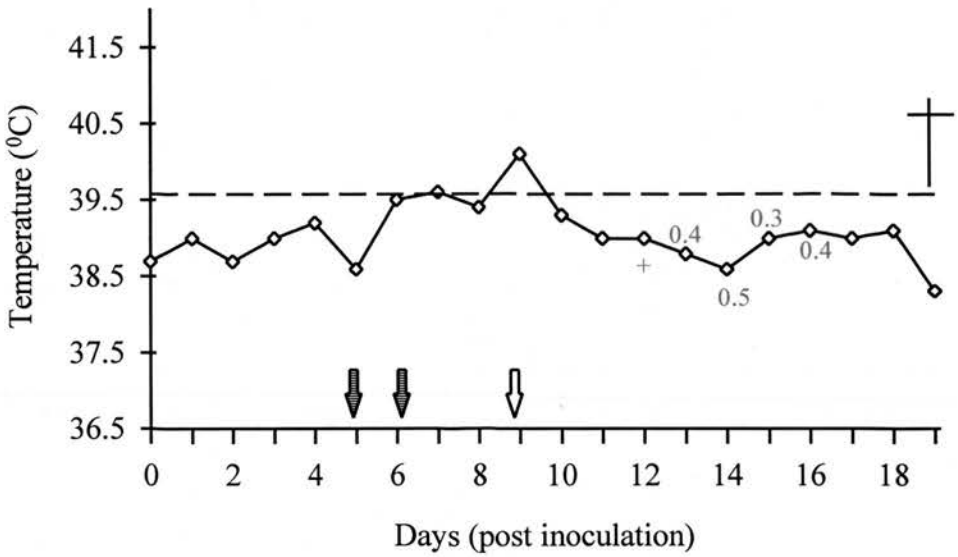
LIMURU											
Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR	Animal	Piros	Ticks	PCR
THI 58/1	- / <0.1	RAm8 (sg)	-	THI 58/X	- / -	RAf2 (sg)	-	GAT 15/X2	- / -	RAf1 (sg)	-
		RAm9 (d)	-			RAf3 (sg)	-				
		RAm10 (d)	-			RAf4 (sg)	-	JET 2/X	- / -	RAm1 (dX3)	-
		RAm11 (d)	-			RAf5 (sg)	-			RAm2 (sg)	-
		RAf1 (sg)	-			RAf6 (sg)	-			RAf1 (sg)	-
		RAf2 (sg)	+			RAf7 (sg)	-				
		RAf3 (sg)	-			RAf8 (sg)	-	JET 2/6	+ / <0.1	RAm1 (dX2)	-
		RAf4 (sg)	-			RAf9 (sg)	-				
		RAf5 (sg)	-			RAf10 (sg)	-	JET 7/1	- / -	RAf1 (sg)	-
		RAf6 (sg)	-							RAf2 (sg)	-
		RAf7 (sg)	-	THI 58/X2	+ / <0.1	RAm1 (sg)	-	JET 18/7	- / -	BOf1 (sg)	-
		RAf8 (sg)	-			RAm2 (sg)	-				
		RAf9 (sg)	-			RAm3 (sg)	-	SHEEP	n.d	RAm1 (dX10)	-
		RAn1 (d)	-			RAf1 (sg)	-			RAm2 (dX10)	-
		RAn2 (d)	-			RAf2 (d)	-			RAm3 (dX10)	-
						RAf3 (d)	-			RAm4 (dX10)	-
THI 58/X	- / -	RAn3 (d)	-			BOm1 (d)	-			RAm5 (dX10)	-
		REm1 (d)	- / -			BOm2 (d)	-			RAm6 (dX10)	-
		REm2 (d)	- / -			BOm3 (d)	-			RAm7 (sg)	-
		REf1 (d)	- / -			BOf1 (d)	-			RAf1 (sg)	-
		REn1 (d)	- / -			BOf2 (d)	-			RAf2 (dX5)	-
		BOf1 (d)	-			BOf3 (d)	-			RAf3 (dX4)	-
		BOf2 (d)	-							RAf4 (dX4)	-
		BOf3 (d)	-	GAT 2/X	- / <0.1	RAm1 (d)	-			RAf5 (sg)	-
						RAm2 (sg)	-			RAf6 (sg)	-
		RAm1 (sg)	-			RAm3 (sg)	-			RAf7 (d)	-
		RAm2 (sg)	-			RAm4 (sg)	-			REm1 (d)	- / -
		RAm3 (sg)	-			REm1 (d)	- / -			REf1 (dX2)	- / -
		RAm4 (sg)	-							REf2 (sg)	- / -
		RAm5 (sg)	-	GAT 4/2	n.d	RAf1 (sg)	-			REf3 (dX5)	- / -
		RAm6 (sg)	-			RAf2 (sg)	-			REf4 (d)	- / -
		RAm7 (sg)	-			RAf3 (dX5)	-			REf5 (d)	- / -
		RAf1 (sg)	-							REf6 (d)	- / -

Table 2E. PCR and bloodsmear examination results from samples collected from the three field sites.

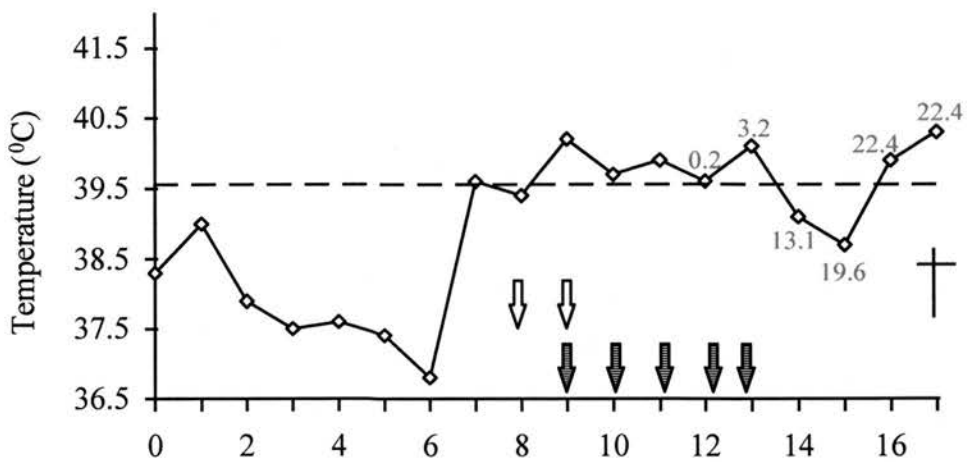
LIMURU					LIMURU				
Animal	*T.parva*	*Theileria*	*Cowdria*	Piro (%)	Animal	*T. parva*	*Theileria*	*Cowdria*	Piro (%)
KANY 8/2	-	n.d	n.d	-	107	-	n.d	n.d	n.d
KANY 8/X	-	n.d	n.d	-	175	+	n.d	n.d	n.d
KANY 8/X2	-	n.d	n.d	-	185	-	n.d	n.d	n.d
KANY 9/1	-	n.d	n.d	<0.1					
KANY 9/X	+	n.d	n.d	-					
KANY 10/2	-	n.d	n.d	-			KITALE		
KANY 11/1	+	n.d	n.d	0.2	1.88	+	+	-	0.2
KANY 11/4	+	n.d	n.d	-	2.82	+	+	-	0.1
KANY 11/5	+	n.d	n.d	0.2	F2A1	+ (3.2Kb)	+	-	0.1
KANY 11/X	-	n.d	n.d	-	F2A2	+ (3.2Kb)	+	-	0.3
KANY 11/X2	+	n.d	n.d	-	F2A3	+ (3.2Kb)	+	-	0.1
THI 21/X	-	n.d	n.d	-	F2A4	+ (3.2Kb)	+	-	0.3
THI 36/2	-	n.d	n.d	<0.1					
THI 36/4	+	n.d	n.d	<0.1			KAKAMEGA		
THI 42/4	-	n.d	n.d	n.d	477	+ (3.2Kb)	+	-	0.2
THI 42/6	+	n.d	n.d	<0.1	481	+ (3.2Kb)	+	-	<0.1
THI 58/1	-	n.d	n.d	<0.1	434	+ (3.2Kb)	+	-	0.7
THI 58/X	-	n.d	n.d	-	435C	+ (3.2Kb)	+	-	0.8
THI 58/X2	+	n.d	n.d	<0.1	441	+ (3.2Kb)	+	-	0.1
GAT 2/X	-	n.d	n.d	<0.1	F4A1	+ (3.2Kb)	+	-	<0.1
GAT 6/3	-	n.d	n.d	-	F4A2	+ (3.2Kb)	+	-	0.2
GAT 6/4	-	n.d	n.d	<0.1	F5A1	+ (3.2Kb)	+	-	0.2
GAT 8/X	-	n.d	n.d	-	F5A2	+ (3.2Kb)	+	-	0.1
GAT 15/X	-	n.d	n.d	-	F5A3	+ (3.2Kb)	+	-	<0.1
GAT 15/X2	-	n.d	n.d	-	F6A1	+ (3.2Kb)	+	-	0.5
JET 2/X	-	n.d	n.d	-	F6A2	+ (3.2Kb)	+	-	0.1
JET 2/6	+	n.d	n.d	<0.1	F6A3	+ (3.2Kb)	+	-	0.1
JET 7/1	-	n.d	n.d	-	F8A1	+ (3.2Kb)	+	-	<0.1
JET 18/1	-	n.d	n.d	-	F9A1	+ (3.2Kb)	+	-	<0.1
JET 18/3	+	n.d	n.d	-	F10A1	+ (3.2Kb)	+	-	<0.1
JET 18/7	-	n.d	n.d	-	F10A2	+ (3.2Kb)	+	-	0.2
NDA	-	n.d	n.d	-	F11A1	+ (3.2Kb)	+	-	0.4

APPENDIX 3

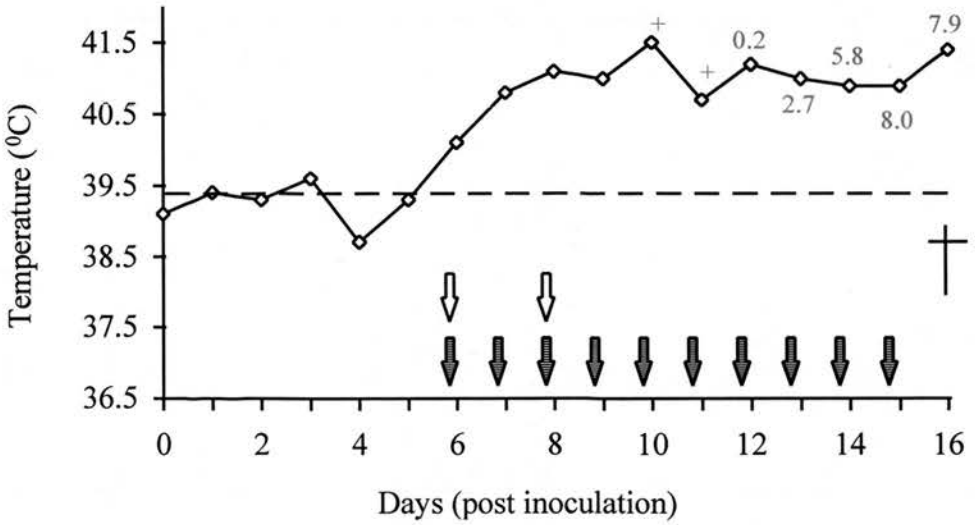
The data included in this section relates to experiments carried out in Chapter 5.



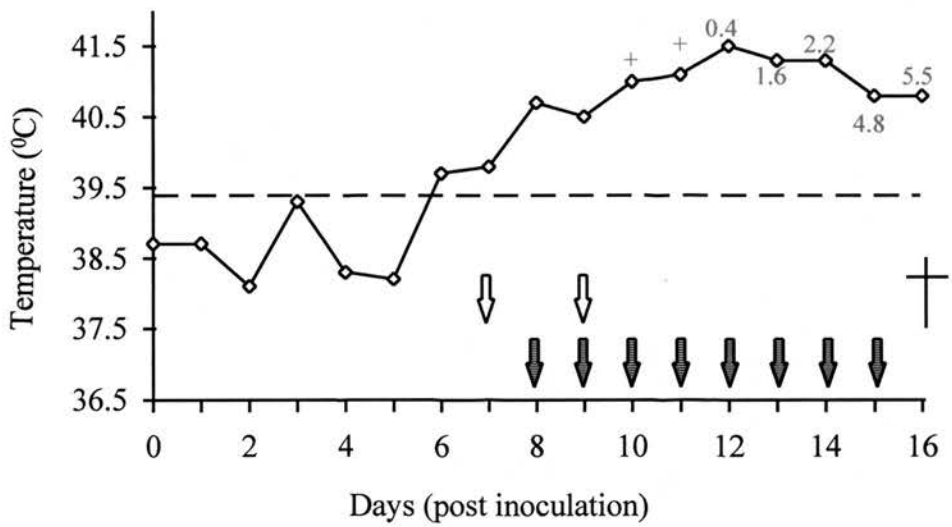
Calf 691A. 0.5ml *T. parva* Muguga Stabilate 71 (1.25t.e - 150 sporoblasts). ⇩ - tick application. ⬇ - Engemycin administration. The red figures represent the piroplasm parasitaemia on those days. Temperatures exceeding 39.5°C (represented by the dashed line) are considered a pyrexia. † - Calf euthanased. + represents piroplasm parasitaemias of less than 0.1% infected erythrocytes.



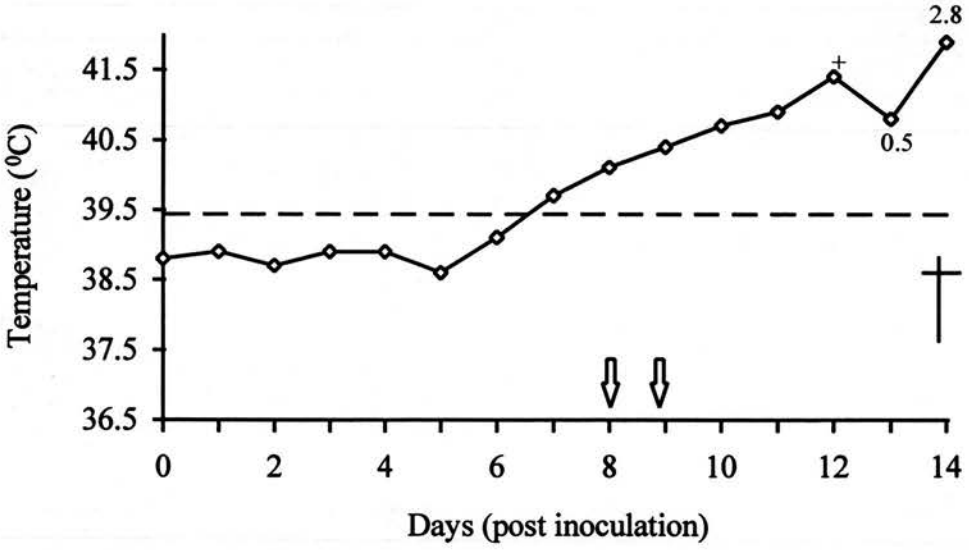
Calf 48A. 0.5ml *T. parva* Muguga Stabilate 71 (1.25t.e - 150 sporoblasts).



Calf 404. 0.5ml *T. parva* Muguga Stabilate 71 (1.25t.e - 150 sporoblasts).



Calf 14500. 0.5ml *T. parva* Muguga Stabilate 71 (1.25t.e - 150 sporoblasts).



Calf 58B. 0.8ml *T. parva* Marikebuni Stabilate 72 (2t.e - 156 sporoblasts).

Table 5A. Ticks were incubated at 28°C. 23°C and 18°C during their nymph-adult moult to investigate the effect on the adult salivary gland infections. The 3 day detachment period is represented by days 1, 2 and 3. % piroplasm parasitaemia (Calf 691A) day 1 - 0.4%, day 2 - 0.5%, day 3 - 0.3%. m / f - male / female. * - refers to incubation temperature after the ticks detached.

detachment day - 28°C*								detachment day - 23°C*								detachment day - 18°C*							
m / f	1	2	3	m / f	1	2	3	m / f	1	2	3	m / f	1	2	3	m/f	1	2	3	m/f	1	2	3
m	0	6	0	f	0	0	89	m	3	65	7	f	0	66	0	m	0	0	0	f	0	0	0
m	48	106	448	f	3	0	0	m	0	69	4	f	11	36	0	m	0	0	0	f	0	0	0
m	0	92	1	f	2	78	2	m	0	209	0	f	14	12	136	m	0	0	0	f	0	0	0
m	69	100	2	f	5	0	0	m	38	80	146	f	2	4	9	m	0	0	0	f	0	0	0
m	0	30	1	f	25	0	1	m	0	296	125	f	16	15	0	m	0	0	0	f	0	0	0
m	51	56	9	f	0	142	51	m	0	241	14	f	22	2	270	m	0	0	0	f	0	0	0
m	25	14	2	f	0	78	22	m	1	23	5	f	0	59	265	m	0	0	0	f	0	0	0
m	0	0	37	f	0	48	37	m	4	0	185	f	0	5	66	m	0	0	0	f	0	0	0
m	10	41	0	f	17	2	4	m	0	33	0	f	29	20	129	m	0	0	0	f	0	0	0
m	0	0	177	f	0	9	0	m	3	8	33	f	0	68	1	m	0	0	0	f	0	0	0
m	8	4	2	f	0	5	10	m	23	5	0	f	0	12	65	m	0	0	0	f	0	0	0
m	11	20	8	f	0	15	0	m	28	1	3	f	44	0	240	m	0	0	0	f	0	0	0
m	0	340	23	f	0	0	67	m	5	33	321	f	25	4	104	m	0	0	0	f	0	0	0
m	3	263	16	f	4	0	27	m	2	2	6	f	38	32	55	m	0	0	0	f	0	0	0
m	7	2	394	f	1	12	0	m	161	8	31	f	4	9	3	m	0	0	0	f	0	0	0
m	14	0	8	f	2	4	18	m	102	66	152	f		4	167	m	0	0	0	f	0	0	0
m	0	0	426	f	20	22	0	m	20	139	24	f		17	56	m	0	0	0	f	0	0	0
m	144	185	0	f	0	0	0	m	2 , 2	4	45	f		17	17	m	0	0	0	f	0	0	0
m	0	0	1	f	3	24	87	m	29 , 2	18	5	f		78	467	m	0	0	0	f	0	0	0
m	0	1	48	f	10		0	m	0 , 2	132	0	f		40	22	m	0	0	0	f	0	0	0
m		0						m	0 , 2			f			1								

Table 5B. 50 Batch A (exposed to *T. parva* as larvae and nymphs)and Batch B (exposed to *T. parva* as nymphs) nymphs were weighed after detaching from calf 404.

Batch A nymphs mass (mg)		Batch B nymphs mass (mg)	
6.5	3.7	12.7	9.1
5.0	3.2	10.7	8.2
8.5	4.8	10.4	7.9
5.5	5.7	10.6	4.8
3.9	4.3	10.2	10.3
6.8	6.0	11.1	6.8
4.8	4.1	9.0	6.7
5.4	7.2	9.9	4.6
4.2	3.9	11.1	6.6
6.1	4.7	8.9	7.5
6.6	5.4	9.7	6.2
4.3	5.2	11.1	7.6
4.5	5.7	9.3	7.8
4.2	4.8	10.9	7.4
3.2	3.8	8.9	12.3
3.5	5.2	7.6	11.2
5.3	6.3	5.3	12.3
5.3	5.6	4.4	8.8
4.7	6.8	9.2	12.8
3.6	5.0	7.0	11.8
4.6	4.5	9.3	10.9
7.1	6.1	8.1	9.8
5.9	5.2	7.3	10.2
8.0	5.8	7.6	8.7
7.2	4.5	6.3	9.7

Table 3C. Comparison of *T. parva* salivary gland infections between Batch A (exposed to *T. parva* as larvae and nymphs) and Batch B (exposed to *T. parva* as nymphs) adult ticks detaching from calf 404. % piroplasm parasitaemia day **1**: + - 0.2. **2**: 0.2 - 2.7. **3**: 2.7 - 5.8. **4**: 5.8 - 8.0. **5**: 8.0 - 7.9.

Batch A - detachment day						Batch B - detachment day					
m/f	1	2	3	4	5	m/f	1	2	3	4	5
m	0	0	0	3	0	m	0	0	182	117	49
m	0	0	0	0	1	m	0	0	110	32	44
m	0	0	0	3	3	m	0	0	34	36	269
m	0	0	0	0	0	m	0	0	0	116	80
m	0	1	0	0	1	m	1	0	55	10	92
m	0	0	0	0	0	m	0	6	0	2	318
m	0	0	2	1	1	m	0	32	1	58	125
m	0	0	0	0	0	m	0	0	2	28	3
m	0	0	1	0	4	m	0	0	0	184	80
m	0	0	0	0	0	m	0	0	26	129	14
m	0	0	0	12	11	m	0	0	3	157	125
m	0	0	0	1	0	m	0	0	13	97	0
m	0	0	0	0	1	m	0	0	3	11	34
m	0	0	0	0	0	m	0	0	66	161	28
m	0	0	0	9	0	m	0	0	12	31	16
m	0	0	0	16	0	m	0	1	6	0	30
m	0	0	0	2	0	m	0	0	0	0	0
m	0	0	0	2	0	m	0	1	3	3	37
m	0	1	0	0	0	m	0	0	0	47	
m	0	0	0	2	0	m	0	8	28	45	
f	0	0	1	0	21	f	0	5	0	218	37
f	0	0	0	0	0	f	0	0	0	6	262
f	0	0	0	0	0	f	0	0	1	3	0
f	0	0	0	0	0	f	0	0	0	20	153
f	0	0	0	0	0	f	0	0	3	0	42
f	0	0	0	0	0	f	0	0	0	0	132
f	0	0	0	0	0	f	0	0	0	10	129
f	0	0	0	0	0	f	0	0	33	93	7
f	0	0	0	0	0	f	0	2	0	225	
f	0	0	0	0	0	f	1	1	0	30	
f	0	0	0	0	0	f	0	1	53	183	
f	0	0	0	0	0	f	0	0	0	9	
f	0	0	0	0	0	f	0	1	1	19	
f	0	0	0	0	0	f	0	0	1	30	
f	0	0	0	0	0	f	0	1	0	15	
f	0	0	0	0	0	f	0	1	0	65	
f	0	0	0	0	0	f	0	0	7	71	
f	0	0	0	0	0	f	0	0	19	229	
f	0	0	0	0	0	f	0	0	5	0	
f	0	0	0	0	0	f	0	0	131	0	

Table 3C (continued). Comparison of *T. parva* salivary gland infections between Batch A (exposed to *T. parva* as larvae and nymphs) and Batch B (exposed to *T. parva* as nymphs) adult ticks detaching from calf 14500. % piroplasm parasitaemia day **1**: 0.4 - 1.6. **2**: 1.6 - 2.2. **3**: 2.2 - 4.8. **4**: 4 .8 - 5.5.

Batch A - Detachment Day					Batch B - Detachment Day				
m/f	1	2	3	4	m/f	1	2	3	4
m	0	0	4	0	m	10	84	38	9
m	0	1	1	0	m	0	42	76	135
m	0	0	0	12	m	0	37	13	96
m	0	0	11	1	m	0	159	4	64
m	0	3	0	1	m	0	134	30	15
m	0	0	0	5	m	1	126	11	51
m	0	2	1	0	m	0	7	27	107
m	0	0	4	1	m	1	88	49	2
m	0	0	0	9	m	0	9	165	34
m	0	0	0	2	m		91	31	4
m	0	38	2	0	m		18	2	3
m	1	19	0	4	m		24	6	73
m	0	11	0	0	m		1	59	181
m	0	0	0	0	m		21	0	10
m	0	0	1	0	m		213	7	44
m	0	13	2	9	m		18	26	278
m	0	0	0	0	m		21	237	166
m	0	0	0	67	m		17	77	355
m	0	0	2	0	m		53	7	32
m	2		3	0	m		266	0	79
f	0	0	0	0	f	0	46	197	157
f	0	0	4	0	f	1	244	52	7
f	0	1	5	0	f	0	118	179	54
f	0	0	0	0	f	0	5	294	7
f	0	1	0	2	f	0	19	112	202
f	0	0	0	0	f	0	58	13	59
f	0	0	0	0	f	1	14	7	12
f	0	2	0	5	f	0	3	2	147
f	0	0	0	0	f	0	96	144	85
f	0	0	0	0	f	0	89	173	1
f	0	0	0	0	f		269	110	1
f	0	0	0	1	f		3	27	4
f	0	0	0	0	f		23	6	37
f	0	0	0	0	f		50	13	292
f	0	0	0	6	f		31	86	63
f	0	4	0	0	f		25	83	97
f	0	5	0	0	f		54	133	118
f	0	2	0	0	f		363	10	160
f	0	0	1	8	f		14	50	29
f	0	3	0	0	f		16	4	48

Table 3D. *T. parva* infections in adult ticks that fed as nymphs on calf 48A. m - male ticks. f - female ticks.

Piroplasm % on Detachment					
3.2 - 13.1		13.1 - 19.6		22.4 - 22.4	
m	f	m	f	m	f
24	162	0	289	7	3
100	24	12	25	103	5
102	73	154	19	0	0
260	87	2	97	12	10
64	23	0	424	1	3
56	88	7	0	14	0
284	32	100	0	0	5
10	266	141	1	1	1
46	204	3	97	2	1
110	48	0	0	1	1
146	77	0	202	1	41
304	50	9	11	0	0
247	212	1	76	7	25
49	74	106	159	0	8
101	295	23	201		1
24	28	0	29		2
314	496	13	137		0
106	97	1	0		0
99	179	10	0		136
	59	0	0		

Table 3E. Quantitative data of *T. parva* forms seen within Giemsa's stained, histological sections of *R. appendiculatus*.

Day 0 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%. Day 2 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
0	d0R880H	1	531/1025		7	80	7				
		2	547/1004		11	46	4				
		3	525/1002	11	151	21					
		4	500/101	3	46	3	1				
	d0R880BD	1	505/1069		398	13					
		2	505/1014	1	113	20	7				
	d0R880AF	1	495/104	2	413						
		2	480/100		16						
		3	500/990		130	7					
2	d2R880K	1	275/1036	4	2						
		2	305/1044			11	8				
		3	320/1026		1						
	d2R880AB	1	272/1039		5	3	1				
		2	306/1026		16	45	2				
		3	301/100		11	57	3				

Table 3E (continued). Day 2 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%. Day 8 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
2	d2R880BF	1	579/1097		7	3					
		2	550/109		1	1					
		3	570/105		43	15	1				
8	d8R880F	1	370/104					0			
		2	397/1037					2			
		3	410/1012					1			
		4	385/993					0			
		5	376/1018					1			
	d8R880AF	1	395/107					1			
		2	370/1047					0			
		3	393/1028					0			
		4	416/1032					0			
	d8R880BB	1	485/1009					0			
		2	510/998					0			
		3	507/970					4			
		4	536/977					0			

Table 3E (continued). Day 9 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%. Day 12 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
9	d9R880B	1	513/1052					33			
		2	494/1042					3			
		3	484/1016					14			
	d9R880AB	1	514/1036					217	13		
		2	518/101					29			
		3	546/1001					64			
		4	548/1028					170	11		
	d9R880BB	1	524/1054					5			
		2	518/1019					199	4		
		3	545/1019					18			
12	d12R880B	1	510/1055					3			
		2	519/1024					9			
		3	485/1035					3			
	d12R880AD	1	525/1081					10			
		2	545/1064					8			
		3	537/1031					2			

Table 3E (continued). Day 12 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%. Day 13 - Tick detachment piroplasm parasitaemia: 22.4 - 22.4%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
12	d12R880BB	1	382/1012					3			
		2	418/997					4			
		3	396/980					8			
13	d13R880F	1	494/1078					0			
		2	495/106					0			
		3	486/1032					0			
		4	463/1023					1			
		5	474/1083					0			
	d13R880AB	1	300/105					10			
		2	274/1052					2			
		3	265/1015					0			
		4	300/100					0			
		5	297/102					1			
	d13R880BD	1	417/1065					2			
		2	417/1041					0			
		3	412/1022					0			
		4	388/1015					1			
		5	378/1038					0			

Table 3E (continued). Day 16 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%. Day 17 - Tick detachment piroplasm parasitaemia: 13.1 - 19.6%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
16	d16N-MR880AH	1	424/1088								
		2	394/1055								
		3	422/1064					1			
	d16N-MR880BE	1	407/1034					2			
		2	362/1015								
		3	375/100								
		4	408/101								
17	d17R880H	1	384/1046					50	1		
		2	412/1034					1			
		3	383/1006								
	d17R880AD	1	291/104					7			
		2	317/1016								
		3	273/984					3		3	
	d17R880BF	1	436/1034								
		2	420/103								
		3	437/1009								
		4	426/991					1		3	

Table 3E (continued). Day 15 - Tick detachment piroplasm parasitaemia: 13.1 - 19.6%. Day 16 - Tick detachment piroplasm parasitaemia: 19.6 - 22.4%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
15	d15R880L	1	380/106					36			
		2	377/1007					64	3		
		3	410/102					25			
		4	390/1034					26			
		5	400/105					7	1		
15	d15R880AF	1	480/104					4			
		2	480/101					66			
		3	500/980					6			
		4	510/100					1			
15	d15R880BH	1	310/106					23	1		
		2	280/105					3			
		3	280/103					7			
		4	290/101					16			
		5	314/1004					1			
16	d16N-MR880H	1	393/1033								
		2	353/1017					2			
		3	378/1007						1		

Table 3E (continued). Day 19 - Tick detachment piroplasm parasitaemia: 13.1 - 19.6%. Day 21 - Tick detachment piroplasm parasitaemia: 13.1 - 19.6%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
19	d19R880H	1	386/1012							2	
		2	410/1002							8	1
		3	388/984								
	d19R880AI	1	558/1069								
		2	577/1047					1			
	d19R880BF	1	517/1036							11	
		2	498/1003					1			
21	d21R880F	1	500/1058					1			
		2	490/1037								
		3	479/1018							1	2
		4	471/1033								
	d21R880AF	1	368/1029							1	
		2	355/1014								1
		3	414/1025								
		4	396/1033								2
		3	396/980								

Tabe 3E (continued). Day 21 - Tick detachment piroplasm parasitaemia: 13.1 - 19.6%.

Day	Slide	Tick	Grid Ref.	Piros in rbc	Free Piros	Gametes in lumen	Zygotes in lumen	Zygotes in gut cells	Kinetes in gut cells	Sporonts	Sporoblasts
21	d21R880BC	1	361/102								
		2	382/976							2	
		3	410/989								
		4	395/1012								

Piros in rbc - intraerythrocytic piroplasms. Free Piros - exoerythrocytic piroplasms.

APPENDIX 4

The data included in this section relates to experiments carried out in Chapter 6.

The reproductive capacity of infected ticks was compared with that of uninfected controls. Two rabbits were used (80B and 83B), and 25 females and 30 males of the control and infected tick batches were placed on each ear. The engorged females were removed after detachment and weighed. Their egg batch mass and hatch rate were later recorded.

Table 4A. (overleaf). Data from control, uninfected female ticks is presented.
Table 4B. (overleaf). Data from infected female ticks is presented. Detach Day - the detachment day after initial female tick application.

Table 4A. Control, uninfected ticks.

Tick Female	Rabbit	Detach Day	Engorged Mass (mg)	Egg Batch Mass (mg)	% Hatch and Survival	Female	Rabbit	Detach Day	Engorged Mass (mg)	Egg Batch Mass (mg)	% Hatch and Survival
1	80B	6	454	277	100	1	83B	7	583	392	99
2		6	200	168	100	2		7	562	191	0
3		7	558	353	100	3		7	569	325	70
4		7	278	214	100	4		7	569	377	100
5		7	518	384	50	5		7	464	306	99
6		7	474	292	90	6		7	422	291	20
7		7	339	242	100	7		7	477	353	100
8		7	543	311	100	8		7	654	401	100
9		7	249	177	5	9		7	633	379	90
10		7	400	295	50	10		7	363	279	5
11		7	396	277	95	11		7	466	327	15
12		7	360	252	100	12		7	486	351	20
13		7	400	284	97	13		7	446	197	85
14		7	311	227	100	14		8	602	398	99
15		7	433	300	100	15		8	465	361	0
16		7	335	240	10	16		8	322	237	5
17		7	331	222	95	17		8	453	307	0
18		7	308	234	60	18		8	477	291	100
19		7	332	233	15	19		8	473	301	0
20		7	507	334	97	20		8	436	306	0
21		7	450	299	5	21		8	615	381	99
22		8	310	214	0	22		8	373	206	0
23		8	202	164	95	23		8	554	367	80
24		8	529	259	100	24		8	407	260	50
25		8	454	-	-						
26		8	241	196	70						

Table 4B. Infected ticks.

Tick Female	Rabbit	Detach Day	Engorged Mass (mg)	Egg Batch Mass (mg)	% Hatch and Survival	Female	Rabbit	Detach Day	Engorged Mass (mg)	Egg Batch Mass (mg)	% Hatch and Survival
1	80B	7	308	205	0	1	83B	7	472	278	99
2		7	244	184	0	2		8	351	238	0
3		7	348	204	0	3		8	282	105	0
4		7	430	277	1	4		8	304	182	99
5		7	405	250	0	5		8	118	103	0
6		7	449	311	0	6		8	517	341	0
7		7	213	115	0	7		8	397	252	0
8		8	430	151	95	8		8	180	145	40
9		8	322	170	60	9		8	477	269	0
10		8	289	106	0	10		8	258	163	0
11		8	362	237	5	11		8	303	149	0
12		8	364	284	0	12		8	193	169	0
13		8	345	243	0	13		8	336	189	0
14		8	415	209	0	14		8	312	224	0
15		8	307	199	0	15		8	199	134	0
16		8	254	-	-	16		8	260	153	0
17		8	343	218	0	17		8	411	214	20
18		8	339	169	0	18		9	368	240	0
19		9	179	108	0	19		9	456	252	99
20		9	146	108	0	20		9	314	192	0
21		9	227	173	0	21		9	281	178	0
22		9	382	267	0	22		10	154	96	0
23		9	270	190	0	23		10	74	45	0
24		10	180	91	40	24		10	89	53	0

APPENDIX 5

The stabilates listed here are those used throughout various experiments, usually associated with the PCR.

DNA samples

- *Amblyomma variegatum* - Uninfected ticks from the CTVM colony.
- *Babesia bigemina* - Stabilate no. 206. Isolated by the NVRC, Muguga, Kenya on 15/8/86.
- *Babesia equi* - A *Rhipicephalus turanicus* tick containing *Babesia equi* was obtained from Dr Henry Kiara from the NVRC, Muguga, Kenya.
- *Cowdria ruminantium* Welgevonden - DNA extracted from 50ml of sheep cell culture. EBs were seen in every field of view on microscopical examination, suggesting a high parasite load. Originally isolated in S. Africa and supplied by Dr F. Jongejan in Utrecht (Supplied by Dr Keith Sumption).
- *Cytocoetes microti* - Culture of rickettsial organisms, originally isolated from a bank vole (Supplied by Dr Keith Sumption).
- *Ehrlichia chaffeensis* - Stabilate received from J. Dawson (Atlanta, USA).
- *Ehrlichia equi* - Infected blood. Blood was taken from a horse suffering from an *E. equi* infection. (Supplied by Dr Keith Sumption).
- *Ehrlichia* 5 stabilate - Tick stabilate prepared by homogenising 25 *Ixodes ricinus*, partially fed nymphs/ml stabilate. DMSO was added to the GUTS as a cryoprotectant. (Prepared by Dr Alan Walker).
- *Ehrlichia* 8 stabilate - Blood stabilate made from a roe deer. A sheep inoculated with the stabilate produced a classical Tick Borne Fever infection with a 25% neutrophil parasitaemia. DMSO was added to the blood as a cryoprotectant. (Prepared by Dr Alan Walker).
- *Rhipicephalus appendiculatus* - Uninfected ticks from the CTVM colony.

- *Theileria hirci* - A variety of cell culture material was DNA extracted depending on what was available at the time (Prepared by Mrs Gwen Wilkie). The material was centrifuged and the cell pellet used for the extraction.
- *Theileria mutans* - GUTS stabilate 88 isolated from Naivasha, Kenya on 20/11/74 by the NVRC, Kenya. *Amblyomma* species were experimentally applied to the field caught buffalo. Infectivity test showed 1/1 naive cattle died of acute *T. mutans* infection upon inoculation. An elevated temperature was seen by Day 13 and a rising piroplasm parasitaemia was produced.
- *Theileria parva* (buffalo derived) - Stabilate 189 from the NVRC, Kenya. Isolated from a buffalo (host species code - BF 5641) on 29/11/84. Infectivity test showed 2/2 naive cattle died of acute ECf upon inoculation.
- *Theileria parva* Lanet

Piroplasms: Infected blood from Calves 76A and 001 (CTVM experiments). The piroplasm parasitaemia did not exceed <0.1%.

Cell culture: Cell culture was generated from PBM separated from infected calves during a patent *T. p* Lanet infection. The cultures sampled were Passage 1 and 2. 10% DMSO was used as a cryoprotectant. (Prepared by Mrs Gwen Wilkie).

- *Tick material*: GUTS stabilate 81. Prevalence and abundance data are unavailable, however, the ticks had very low infections. The stabilate was diluted to 1 t.e/ml and the cryoprotectant was 7.5% Glycerol. (Prepared by Mrs Gwen Wilkie).
- *T. parva* Lawrenci - Stabilate 193 from the NVRC, Kenya. Isolated from a buffalo (host species code - BF 5641) on 11/05/85.
- *Theileria parva* Marikebuni

Piroplasms: 2.8% *T. p* Marikebuni piroplasm infected blood.

Cell culture: A variety of cell culture material was DNA extracted depending on what was available at the time (Prepared by Mrs Gwen Wilkie). The material was centrifuged and the cell pellet used for the extraction.

Tick material: GUTS stabilate 72. Tick batch showed a 95% infection with an average of 78 infected acini per tick. The stabilate was diluted to 2.5 t.e/ml and the cryoprotectant was 7.5% Glycerol. (Prepared by Mrs Gwen Wilkie).

- *Theileria parva* Muguga

Piroplasms: A protocol for purifying piroplasms can be found in Chapter 3.

Cell culture: A variety of cell culture material was DNA extracted depending on what was available at the time (Prepared by Mrs Gwen Wilkie). The material was centrifuged and the cell pellet used for the extraction.

Tick material: GUTS Stabilate 71. Tick batch showed a 95% infection with an average of 121 infected acini per tick. The stabilate was diluted to 2.5 t.e/ml and the cryoprotectant was 7.5% Glycerol. (Prepared by Mrs Gwen Wilkie).

- *Theileria taurotragi* - GUTS stabilate made from *R. appendiculatus*. Supplied by the NVRC, Kenya.

APPENDIX 6

- **Acid citrate dextrose** (ACD - anticoagulent):

80mM disodium hydrogen citrate ($Na_2HC_6O_71^1/_2H_2O$)
140mM D(+)Glucose ($C_6H_{12}O_6$)
(Made in distilled water)

- **Casein solution** (10 x stock solution from Vector Laboratories. Cat No: SP-5020) - diluted 1:10 for use in distilled water. 1 x Casein solution: 0.5% (w/v) casein, phosphate buffered saline, 0.1% (v/v) Tween-20.

Vector Laboratories, Peterborough, England.

- **Karnovsky's Fixative**

2.5% gluteraldehyde
2% formaldehyde
(Made in 0.1M phosphate buffer*).

* 0.2M phosphate buffer stock solution, made in distilled water:
1.5 x 10^{-3}M calcium chloride anhydrous ($CaCl_22H_2O$)
5.6 x 10^{-3}M dihydrogen orthophosphate ($NaH2PO_4 \cdot H_2O$)
1.4 x 10^{-2}M disodium hydrogen orthophosphate (Na_2HPO_4)

- **NBT/BCIP stock solution** (Cat No: 1681 451): Boehringer Mannheim

Nitro blue tetrazolium chloride (NBT) 18.75 mg/ml, 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) 9.4 mg/ml in 67% DMSO (v/v).

Roche Diagnostics Ltd. East Sussex, England.

- **Saline-sodium citrate buffer** (SSC) (x 20 stock solution)

3M sodium chloride (NaCl)
0.3M sodium citrate ($C_6H_5Na_3O_7 \bullet 2H_2O$)
(Made in distilled water, adjust to pH 7.0 with NaOH).

- **Tris buffered saline** (TBS pH 7.5)

0.14M sodium chloride (NaCl)
2.7 x 10^{-3}M potassium chloride (Kcl)
2.5 x 10^{-2}M Tris base ($C_4H_{11}NO_3$)
4.2 x 10^{-5}M phenol red ($C_{19}H_{14}O_5S$)
(Made in distilled water, adjust to pH 7.5 with HCl).

- **Tris borate** (TBE) (x 5 stock solution)

0.45M Tris base ($C_4H_{11}NO_3$)
0.45M boric acid (H_3BO_3)
0.01M EDTA, pH 8.0 ($C_{10}H_{16}N_2O_8$)
(Made, and diluted for use in distilled water)

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ORIGINAL PAPER

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Use of the polymerase chain reaction for identification and quantification of *Theileria parva* protozoa in *Rhipicephalus appendiculatus* ticks

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Abstract The polymerase chain reaction (PCR) was adapted for detection of *Theileria parva* sporoblasts in *Rhipicephalus appendiculatus* ticks by comparison with staining of histological preparations of ticks with methyl green and pyronin (MGP). Two 32mer primers (IL174 and IL179) were used to amplify *Theileria parva* (Muguga isolate) DNA from the TPR 1 region of the genome by the PCR. Detection of *T. parva* was carried out with dissected salivary glands and whole ticks preserved in ethanol. Adult ticks which fed as nymphs on a *T. parva* infected calf were used in three experiments. Firstly, 70 whole ticks divided into 7 batches representing the rising and falling parasitaemia of the calf were used to show that detection of infection by the PCR was significantly correlated with MGP staining. Secondly, 120 dissected ticks were used from 4 different batches representative of the overall infection profile within the ticks to show a high correlation between PCR quantification within tick salivary glands and MGP count data of the paired gland. Thirdly, 120 ticks were used in batches selected for high and low infections. Bloodmeal contaminants from partially fed adult ticks, present in 60 out of the 120 ticks used, did not inhibit the PCR amplification of *T. parva* DNA. This experiment also showed a great increase in infection detection in partially fed batches of ticks compared to the untreated batches.

Introduction

Theileria parva is a haemoprotozoan parasite transmitted by the tick *Rhipicephalus appendiculatus*, causing East Coast fever of cattle. The dynamics of *Theileria* flow through the tick populations is poorly understood. The infection and treatment method of vaccination currently used to immunize cattle against *Theileria parva* (Cunningham et al. 1989) will require monitoring of the epidemiological effects of introducing live vaccine derived *T. parva* into cattle and thus ticks. Such monitoring should include routine assessment of infection in ticks. However, the histological methods for this (Blewett and Branagan 1973; Walker et al. 1979) require live ticks to be returned to the laboratory, and they are not species specific. The PCR can overcome this by using ethanol fixation in the field and species specific probes. The PCR has been adapted for *Theileria annulata* in *Hyalomma* ticks (De Kok et al. 1993) and *Cowdria ruminantium* in *Amblyomma* ticks (Peter et al. 1995). We adapted standard PCR for use in an epidemiological survey of East Coast fever which should use unfed, questing ticks on vegetation and ticks attached to, and partially fed on cattle, with preservation of the ticks in ethanol in the field.

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Materials and methods

Calf

A 5 month old male Friesian calf was infected with *Theileria parva* (Muguga isolate) stabilate; 0.25 tick equivalent was injected subcutaneously above the right parotid lymph node. The *T. parva* piroplasm parasitaemia of the calf was recorded daily from smears made from blood samples taken from the calf. Experimental animals were used under the sanctions of the Animals (Scientific Procedures) Act of the United Kingdom.

Ticks. 1000 *Rhipicephalus appendiculatus* nymphs (isolated from Muguga, Kenya) were applied to each of two earbags and a neck patch which were inspected daily. Unattached ticks were removed on day 1. Detached, engorged nymphs were stored at 28 °C, 85% relative humidity, 14 hour light/10 hour dark for 28 days and thereafter at 18 °C.

Salivary Gland Staining

Salivary glands of the ticks were examined to detect the sporoblast stage of *Theileria* using the methods of Walker et al.(1979), with the dissected glands being teased out and dried on a microscope slide

prior to staining. We needed to use unfed ticks representative of field collections of questing ticks. For such ticks we prefer the MGP method to the Feulgen method (Blewett and Branagan 1973) which is effective for sporoblasts in ticks which are incubated or partially fed to stimulate the development of the sporoblasts. Stained glands were examined using 250 × magnification.

PCR method

Sample material was placed in 180 µl of tissue lysis buffer (Buffer ATL-QIAGEN Ltd-Surrey, England) and digested using the QIAamp Tissue Kit (QIAGEN Ltd) and the DNA was eluted in a final volume of 200 µl of de-ionised water. The 100 µl PCR reaction mixture contained two 32mer primers, which flank a 405 bp region on the TPR 1 gene for *T. parva* [Muguga isolate; Bishop et al. 1992; 10 µl primer IL174 (1 µM) and 10 µl primer IL179 (1 µM)], 51.5 µl distilled water, 10 µl deoxyribonucleotide triphosphate (200 µM each dNTP), 10 µl PCR buffer (10X-Gibco), 3 µl MgCl (1.5 mM), 0.5 µl of *Taq* DNA polymerase (5 u/ml-Gibco) and 5 µl of template. Each sample was overlaid with 100 µl of mineral oil (Sigma-Dorset, England). Amplification took place in a thermal cycler (Hybaid-Middlesex, England) as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min. 13 µl of the amplified products were run on a 1.6% agarose gel and visualised using an ultra violet-light transilluminator.

Experimental format

The first experiment was a comparison of salivary glands dissected from paired ticks representing the population which fed over a range of piroplasm parasitaemias, using MGP for 1 tick of each pair and the PCR for the other to assess the prevalence of infection by both methods. 140 ticks were used, 70 for MGP and 70 for the PCR. 7 groups of ticks were selected, with 5 males and 5 females in each group from a rising and falling parasitaemic profile. The values selected were rising to < 0.1%, 0.2%, 0.8%, 2.1% and falling to 0.8%, 0.1% and < 0.1%. Those used for MGP had both glands removed and stained, which also allowed a direct comparison of gland to gland infection levels as has been described by Chen et al. (1991). The glands to be used for PCR were removed and placed directly in 180 µl of Buffer ATL. The ticks were stuck in wax in separate Petri dishes and a new scalpel blade was used for each dissection. This was to minimise cross contamination between ticks. Forceps and needles were wiped with a clean tissue, dipped in 100% ethanol and flamed after each use.

The second experiment was to compare the MGP and PCR methods on paired salivary glands removed from individual ticks to compare both prevalence and density of infection using both methods. 120 ticks were used divided into 4 groups of 30 (15 males, 15 females). Batches were chosen on the basis of the double gland count carried out in the previous experiment. The batches chosen were: uninfected, rising to < 0.1%, rising to 0.2% and falling to 0.8%. It was thought that these batches would offer a well graded range of infectivity. The object of the experiment was to compare the MGP record of infection in one salivary gland with the presence and subsequent brightness of the band detected on the agarose gel after the PCR of the opposite gland. The ticks were stuck in wax in separate Petri dishes as described in experiment 1. The gland to be used for PCR was removed first (to avoid potential contamination from the other gland) and placed directly in 180 µl of Buffer ATL. The glands to be stained were fixed on individually marked slides and dealt with as described previously. The salivary glands were placed into 1.5 ml microfuge tubes and heated in a water bath at 55 °C for 1 hour. The rest of the digestion procedure is as described in the QIAamp protocol. Notation on the microscope slide and the PCR tube number enabled a comparison of the results of light microscopy with those of the PCR.

After the salivary glands were stained, one author covered the markings on the slides with labels so they could not be identified. They were then thoroughly randomised and had a code from 1–120 assigned. The counting was done by a separate author who had not previously seen the slides. After the count data had been tabulated along with the code number, a third author removed the label and on a separate data sheet recorded the slide number with the code. The count data was matched with the slide from which it was extracted.

The intensity of the PCR product band was recorded on a direct visual ranking scale from 0 (nothing seen) to 5 (large, bright band seen).

The third experiment was to compare the effectiveness of the PCR when applied to unfed ticks or to partially fed ticks representative of field collections. *Theileria* infected ticks which have fed for up to five days will have much more *Theileria* DNA than when unfed (Shaw and Young 1995), but the extra tick tissue and cattle blood could interfere with the test. 120 ticks were used from 2 batches for the PCR process. 60 (30 males, 30 females) ticks from the rising to 0.2% parasitaemia group and 60 from the rising to 2.1% group. 30 (15 males, 15 females) ticks from each batch were used untreated and dealt with as described previously. The two batches of 30 ticks to be partially fed were placed separately on rabbit ears and allowed to feed for 4 days. Then all the ticks were placed in 100% ethanol and allowed to fix for a minimum of 2 days. They were then bisected in individual Petri-dishes using a new scalpel blade each time and both halves placed together in 180 µl of Buffer ATL. The bisection was to allow maximum exposure of tissue to the digestive enzymes. The rest of the digestion and amplification proceeded as described in the previous experiments. The amplified DNA was run on 1.6% agarose gels in batches of 15 (same sex) for comparison between groups. 200 µl of rabbit blood was digested using the QIAGEN Blood Kit and tested by the PCR before and after tick application to ensure there was no cross-reactivity with *T. parva* DNA and that *T. parva* DNA was not circulating within the bloodstream.

Because of the necessity to discriminate between *T. taurotragi* and *T. parva*, *T. taurotragi* DNA was used with the same primers. The reaction was carried out as described above. After amplification and run out on a 1.6% agarose gel, *T. taurotragi* showed two bands of higher molecular weight compared with the one band that is characteristic of *T. parva* (Muguga isolate; unpublished data).

Results

Figure 1 shows the PCR and MGP results from the comparison of dissected glands from paired ticks from the first experiment. The PCR appears to detect more infection than MGP when the parasitaemia is dropping, but a Mann-Whitney U-Test performed on the data revealed that there is no significant difference between the medians of the data sets [critical value = 6, U = 18; $P < 0.05$ (two-tailed test)]. Examination of both stained glands from 70 ticks revealed 28 to be uninfected and out of the 42 infected, 14.3% revealed an imbalance in their gland infection. MGP detected 42/70 infections, while the PCR detected 37/70. A statistical analysis on the results showed that any differences between the two detection methods was not significant (Mann-Whitney U-test, 95% confidence level).

In the second experiment the band intensities on the gel showed a sufficient range to allow an arbitrary grading. A scale of 0 (no infection detected) to 5 (large infection status) was decided upon as can be seen in Fig. 2.

The detection of infection by the PCR was slightly greater than the results from MGP (4.1%-not statistically significant). Figure 3 shows a graph correlating the

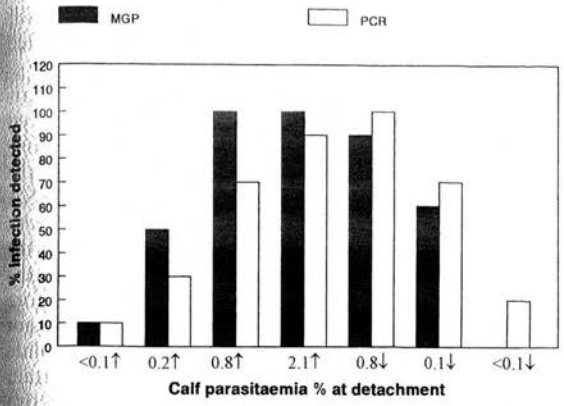

Fig. 1 Infection detection comparison between MGP staining and the PCR

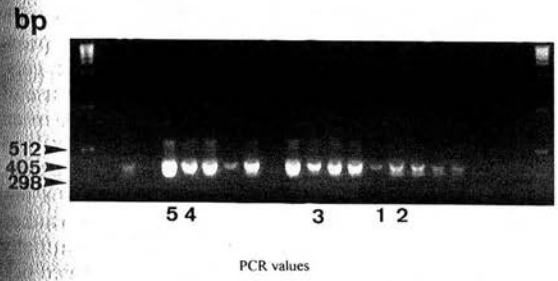

Fig. 2 Range of band intensities and their ranked values

MGP count data with the values assigned to the PCR band intensities. The Kendall Rank Correlation gives a value of 0.75 at $P < 0.001$. It should be noted that graphing data from Kendall Rank Correlations does not allow a line of best fit to be inserted.

In the third experiment it was found that rabbit DNA present in the tick bloodmeals was not amplified and the additional protein and lipids in the partially fed ticks did not appear to inhibit the amplification as previously described (Johnson et al. 1992). The batch of ticks from a calf parasitaemia showed a great increase in the intensity of the amplification after the ticks were partially fed. The average intensity of infection from the PCR ranking showed females to have slightly lower infection levels than males with a mean rank of 1.27 as opposed to 1.47. The numbers infected were 10/15 and 9/15 respectively. Upon feeding, all 15 ticks in both sexes were detected as positive for infection with the males having a higher intensity rating of 3.73 as opposed to 2.40 for the females. The batch of ticks from a calf parasitaemia rising to 2.1% showed a slightly different pattern. The untreated males had a lower infection intensity than the females with a figure of 2.27 as opposed to 2.73. After feeding however, the males had a mean rank of 4.73 as compared with 3.8 for the females, showing a greater

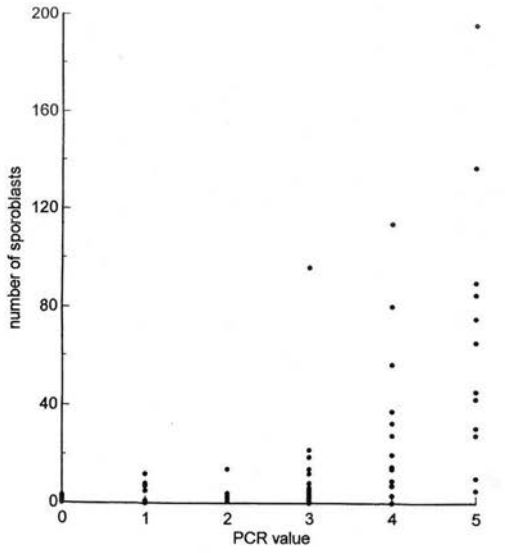

Fig. 3 Correlation between PCR ranks and sporoblast count data for paired glands. A total of 120 ticks were used, but the number of ticks represented by each PCR rank varied quite markedly. PCR 0–59 ticks, PCR 1–13 ticks, PCR 2–7 ticks, PCR 3–14 ticks, PCR 4–15 ticks and PCR 5–12 ticks

percentage increase as before. The rabbit blood showed no amplification before or after tick application. Figure 4 shows the results from untreated and partially fed male ticks.

Discussion

Studies have been conducted before using molecular probes to detect *Theileria parva* in *Rhipicephalus appendiculatus* (Chen et al. 1991). Removal of the salivary glands was required however, as was partially feeding the ticks to increase the amount of DNA available. The collection of field material for epidemiological purposes however, is most conveniently ethanol preserved foregoing this option. The technique described here applies the PCR to whole, ethanol preserved ticks ranging from untreated to 4 day partial fed batches typically available at field collections. It also applies the PCR to ticks of low infection prevalence and intensity typical of field infections and to high prevalence and intensity.

Theileria taurotragi is a non-pathogenic species which can also be transmitted by *Rhipicephalus appendiculatus*. The need to discriminate between this and *T. parva* is critical but is made difficult at a molecular level because they share so much sequence homology (Bishop et al. 1994). With the primers used in these experiments it is possible to readily distinguish between the two species on the basis of the band profile generated on a gel.

It has been shown before that it is possible to detect one sporoblast in an infected salivary gland using the

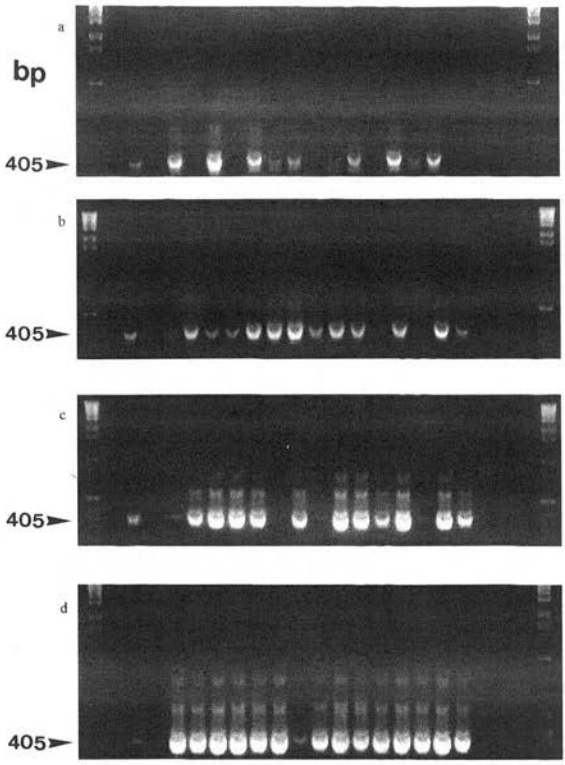

Fig. 4 a) 15 untreated male ticks which dropped off at 0.2% parasitaemia. b) 15 partially fed male ticks which dropped off at 0.2% parasitaemia. c) 15 untreated male ticks which dropped off at 2.1% parasitaemia. d) 15 partially fed male ticks which dropped off at 2.1% parasitaemia

PCR (Bishop et al. 1992). Although we did not specifically isolate one sporoblast in an otherwise uninfected gland, the PCR did prove to be capable of detecting parasites in samples from ticks in which only one sporoblast was observed by microscopic examination in the paired salivary gland as has been found in previous studies (Chen et al. 1991). The ticks used for the amplification were untreated and therefore had undeveloped sporoblasts. These were often very small, particularly compared to those in incubated or partially fed ticks and so shows the sensitivity of the PCR technique to detect *T. parva* from within much greater amounts of tick DNA. Microscopical examination of MGP stained glands frequently reveals variation in the relative maturity of sporoblasts in glands from untreated and partially fed ticks. With untreated ticks however, this variation in the salivary glands tends to be more pronounced. It is common in these cases to see the majority of sporoblasts in primordia, but, in a few cases there are some which are substantially larger and semi-mature. These sporoblasts contain much greater amounts of *T. parva* DNA and can therefore account for PCR values that are higher than would be expected for the actual number of sporoblasts present as determined by the MGP count from the paired gland.

The comparison of the PCR in untreated and partially fed ticks showed a large increase in the amplified products from the partially fed batches, but it also showed an increase in the prevalence of infection in the tick groups. Figure 4 shows a typical partial-feed DNA profile. All the values cluster around ranks 4 to 5 except for a few with a 1 rating. The numbers of ticks with the higher levels of infection correlate very closely with the levels of infection detected in the same batch before partial feeding. We tested the rabbit blood and could not detect parasite DNA within the circulation, so speculate that the ticks giving a value of 1 could actually contain contaminative sporozoites in their gut from infective ticks feeding beside them. A similar phenomenon has been demonstrated previously with the transfer of tick-borne encephalitis virus in *Rhipicephalus appendiculatus* ticks (Labuda et al. 1993). This effect could lead to an overestimation of infection levels in studies dealing with tick batches of varying infection feeding in close proximity to each other as tends to occur on cattle ears. Future work in this area could potentially make use of RNA amplification techniques rather than DNA in an attempt to detect live sporozoites in salivary glands as opposed to piroplasms (or sporozoites) in the gut of which the majority should get digested within a few days. It has to be borne in mind that a PCR ranked value from an untreated tick represents a substantially greater number of sporoblasts than a fully engorged tick with the same value. This is because the actual amount of DNA in a partially fed tick is greatly amplified and therefore fewer sporoblasts can represent the same DNA quantity as a larger number in unfed, unincubated ticks. Thus, it is essential to consider the physical condition of the tick used in the processing and relate this to the result obtained.

We conclude that the described method works for assessing infection prevalence and density of infection in ethanol preserved, untreated or partially fed ticks typical of field collections.

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A PCR-based Field Evaluation of *Theileria* Infections in Cattle and Ticks in Kenya[a]

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ABSTRACT: *Theileria parva* is a hemoprotozoan parasite responsible for causing East Coast fever in east and central Africa. The vaccine currently available is an "infection and treatment" procedure which involves the injection of live sporozoites followed by drug therapy to prevent clinical illness. Before introducing potentially new strains of parasite into an area, however, it is crucial to check the disease situation in the field first. We looked at three different areas in Kenya: Limuru in which many cattle have already been vaccinated and Kitale and Kakamega which so far have not been vaccinated. Genus and species specific primers were used to test for the presence of *Theileria* species in blood and tick samples collected from the three areas. Limuru showed a cattle and tick infection prevalence of 27% and 2.3% respectively with *T. parva*. Kitale showed a cattle and tick infection prevalence of 100% and 14.2% respectively while Kakamega showed 100% and 0% respectively. Reasons for variations between areas involving vaccination status and epidemiological data are discussed.

Theileria parva is a hemoprotozoan parasite transmitted by the brown ear tick *Rhipicephalus appendiculatus,* causing East Coast fever (ECF) of cattle. The disease is of great economic importance to the countries affected.[1,2] The 'infection and treatment' method of vaccination currently used to immunize cattle against *T. parva*[3,4] will require monitoring of the epidemiological effects of introducing live vaccine–derived *T. parva* into cattle and ticks. The PCR has previously been used to characterize and quantify *T. parva* infections in blood and ticks.[5,6] We describe here the use of these techniques and applications on field collected blood and tick samples. We selected three different areas within Kenya; Limuru, Kitale and Kakamega. An ECF vaccine using the *T. parva* Marikebuni stock has been used to vaccinate cattle in Limuru, while the areas we collected from in Kitale and Kakamega remained unvaccinated. The infection prevalences in cattle and ticks were markedly different between areas, as were the species of *Theileria* found. We discuss the results and the implications these could have for future vaccination trials in these areas.

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MATERIALS AND METHODS

Study Area

Limuru is located in close proximity to the National Veterinary Research Centre (NVRC, which is in charge of ECF vaccination in Kenya) at Muguga and has been used as an experimental area to try the ECF vaccine. It is mainly *Bos taurus* breeds in this area belonging to small-holder dairy farmers. Sixty percent of the animals we sampled in this area had been vaccinated. Kitale is stocked mainly with exotic breeds, while the farms we visited in Kakamega were entirely stocked with native species such as zebu-borans. Kitale and Kakamega are two areas that are being considered for vaccination trials in 1997. FIGURE 1 depicts a map showing the positions of the three areas. Tick control was practiced regularly in Kitale with some animals being dipped twice a month and others being treated with spray-on acaricide (Triatix-Coopers). No tick control was being practiced in Kakamega. The number of farms visited and samples taken in each area can be seen in TABLE 1.

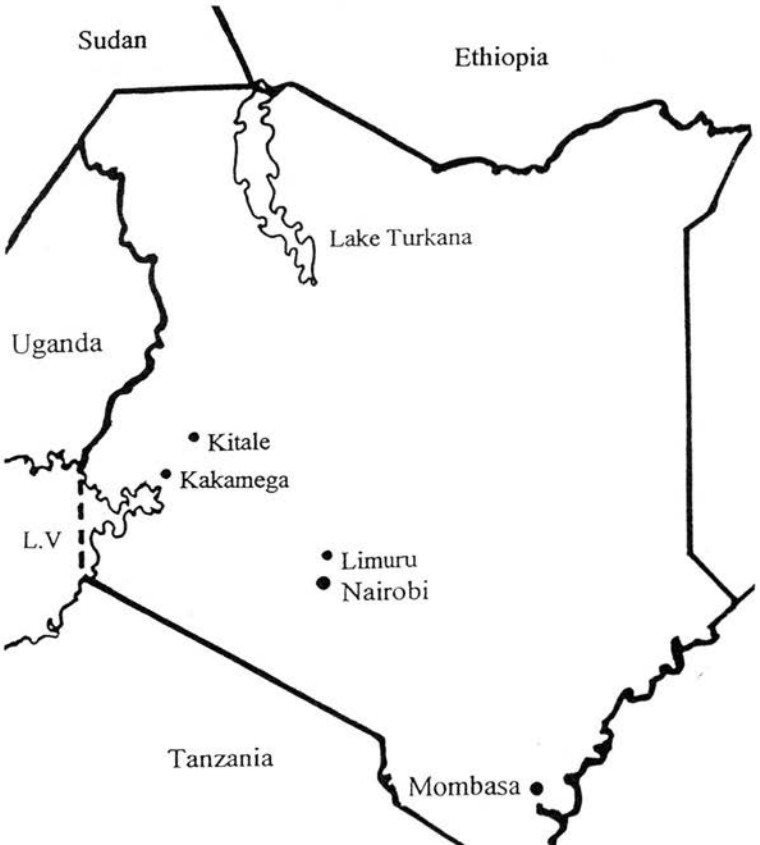

TABLE 1. Data on the Number of Farms Visited and Samples Taken

Area	No. of Farms Visited	No. of Blood Samples Taken	No. of Ticks Collected				Total No. of Ticks
			R.app	*R.eve*	*A.var*	*B.dec*	
Limuru	17	33	308	33	0	36	377
Kitale	4	6	44	10	19	21	94
Kakamega	11	16	133	2	122	28	285

R.app = Rhipicephalus appendiculatus; R.eve = Rhipicephalus evertsi; A.var = Amblyomma variegatum; B.dec = Boophilus decoloratus.

Blood Samples

Blood samples were collected in 10 ml vacutainer tubes that contained approximately 1 ml of acid citrate dextrose (ACD) anticoagulant. The tubes were stored in wet sand for a maximum of 2 days until we returned to the NVRC. DNA was extracted from 200 µl volumes of blood using a QIAamp Tissue extraction kit and protocols from Qiagen Ltd. (Surrey, England). Blood smears were made by fixing the smear in 100% methanol for 3 min and then placing them in 5% Giemsa stain (Merck, Dorset, England) for 40 mi. They were then gently rinsed with Gurr Giemsa buffer (Merck, Dorset, England) and after drying, examined under a light microscope at ×1000 magnification.

Tick Samples

Animals were carefully examined over their whole bodies for the presence of ticks. Ticks were removed and placed in tubes which were then sealed with a cotton wool bung and stored in wet sand until we returned to the laboratory. At the laboratory they were identified and split into different groups depending on species, sex and their physical condition. Semi-engorged live ticks were stored at 18°C, 85% relative humidity until they were dissected to allow removal of the salivary glands. The procedure was performed as described previously.[7] Live-unfed ticks were placed in ear bags on New Zealand White rabbits and allowed to feed for 4 days before being removed. This was to allow any potential salivary gland infection to mature after which their salivary glands were dissected and used for the PCR. Dead ticks were placed in 100% alcohol (EtOH) and stored until there was time to process them. Tick material to be DNA extracted was placed in 180 µl of tissue lysis Buffer ATL (QIAamp) and the procedure followed was as described by the manufacturer (Qiagen Ltd.). Dead ticks were always bisected to allow maximum exposure of their soft, internal tissues to Proteinase K and Buffer ATL prior to DNA extraction.

PCR Procedure

Two primer sets were used to amplify *Theileria* sequences. The sequences and programs used can be seen in TABLE 2. Both primer sets, IL194 / IL197 and 989 / 990 have been described previously.[5,8] DNAs from different species were used to determine the

TABLE 2. Primer Sequences and PCR Program Information Used

Primers	Sequences—5′ → 3′	Area of Genome	Band Size	PCR Program
IL194 / IL197	194—ATA TAT CCA GCC ATA GCT CCT GGA ATG ATT GT 197—TAC AGC CAA TGA GAT CTC ATG ACA CAT ATA GA	TPR1 *(T. parva)*	402bp—405bp	2mins 94°C (1 cycle) 1min 94°C,1min 65°C,1min 72°C (30 cycles) 10mins 72°C (1 cycle)
989 / 990	989—AGT TTC TGA CCT ATC AG 990—TTG CCT TAA ACT TCC TTG	sr RNA *(Theileria)*	1.09 Kb	2mins 94°C (1 cycle) 1min 94°C,2mins 55°C,1min 72°C (30 cycles) 10mins 72°C (1 cycle)

TABLE 3. Specificity of Primer Sets

Primers	Species/Stocks Amplified	Species/Stocks **not** Amplified
IL194 / IL197	*T. p* Muguga, *T. p* Marikebuni *T. p* Lanet, *T. p* Lawrencei *T. p* (buffalo derived) *T. p* Boleni, *T. taurotragi*	*T. annulata*, *T. hirci* *T. buffeli*, *T. velifera* *T. mutans*, *B. bigemina* *B. bovis*, *C. ruminantium* *E. chaffeensis* Bovine *R. appendiculatus* (tick) *A. variegatum* (tick)
989 / 990	*T. annulata*, *T. buffeli* *T. taurotragi*, *T. hirci*	*T. p* Muguga, *T. p* Marikebuni *T. p* Lanet, *T. p* Lawrencei *T.* mutans, *C. ruminantium* *E. chaffeensis*, Bovine *R. appendiculatus* (tick) *A. variegatum* (tick)

T. p = Theileria parva; C. ruminantium = Cowdria ruminantium; E. chaffeensis = Ehrlichia chaffeensis; B. bigemina = Babesia bigemina; B. bovis = Babesia bovis.

6× loading buffer (Sigma—Dorset, England) and were run on 1.6% agarose gels containing 4.8×10^{-4} mg/ml ethidium bromide and visualized using an ultra violet-light transilluminator.

RESULTS

Primer Specificity and Amplification

Primer specificity can be seen in TABLE 3. A high molecular weight (hmw) band of around 3.2 kb was seen using the TPR1 region primers in 67% of the blood samples from Kitale and 100% of the blood samples from Kakamega as opposed to the usual 402/405 bp band normally associated with *T. parva* recognition with these primers (see TABLE 2). This band was not present in any of the samples amplified from Limuru. Uninfected bovine blood used as controls did not show any amplification with the primers.

Blood Smear and PCR Correlation

The detection of *Theileria* infection in blood samples between the PCR (using both primer sets) and the blood smears (recording piroplasm infected erythrocytes) was highly correlated. Overall, out of 55 blood samples taken, there was an 84% agreement between amplification with the PCR and piroplasm detection in the blood smears. In 11% of cases the PCR detected *Theileria* infections while no piroplasms were seen in the smear, and *Theileria* piroplasms were seen in 5% of smears when infection was not detected in the same sample by the PCR.

Infection Prevalences in Samples

Tick species and numbers collected can be seen in TABLE 1. TABLE 4 shows the infection results for blood and ticks from the three areas. All of the *R. evertsi* samples

TABLE 4. Infections Detected by the PCR in Collection Areas

Area	Primers	Parasite / Species	% Blood +ve (n)	% Tick +ve *R. app*	*A. var*	*B. dec*
Limuru	IL194 / IL197	*T. parva*	43.5 (33)	2.3	1.3	0
	989 / 990	other *Theileria* sp.	0 (33)	0	0	0
Kitale	IL194 / IL197	*T. parva*	33 (6)	11.5	0	0
	989 / 990	other *Theileria* sp.	100 (6)	11.5	10.5	0
Kakamega	IL194 / IL197	*T. parva*	0 (16)	0	0	0
	989 / 990	other *Theileria* sp.	100 (16)	0.4	0	0

R. app = Rhipicephalus appendiculatus; A. var = Amblyomma variegatum; B. dec = Boophilus decoloratus; n = number of samples.

were found to be negative for infection. DNA from blood samples was amplified by the authors in Kenya and in our laboratory in Edinburgh and gave the same results each time.

In Limuru, 43.5% of cattle tested were detected as being *T. parva* positive. Out of these, 6 out of 15 (40%) unvaccinated and 8 out of 17 (47%) animals vaccinated against ECF with a *T. parva* Marikebuni stabilate were detected as being *T. parva* positive. Two of the cattle and one *R. appendiculatus* in Kitale were detected as having a mixed infection with *T. parva* and other *Theileria* species as a hmw band was detected upon amplification with the TPR1 primers. One *R. appendiculatus* tick was found to amplify a very faint band when primers 989/990 were used.

DISCUSSION

Primers IL 194 and IL197 amplify a single band around 402/405 bp for *T. parva,* depending on the stock and a double band around 420 and 450 bp for *T. taurotragi* (data not shown). The 402/405 bp band size has been the only one reported for the TPR1 region primers to the authors' best knowledge and the only one we have seen under experimental conditions in our laboratory using a wide variety of *Theileria* species (see TABLE 3). It was surprising therefore, to see the 3.2 kb band in the blood samples from Kitale and Kakamega. We assumed the band came from other species of *Theileria* as '*Theileria*-like' piroplasms were clearly visible in the blood smears from these animals. To investigate this further we used primers 989/990 which had previously been reported as being general *Theileria* primers by Allsopp *et al.*[8] Despite the fact that we were unable to duplicate the results by the previous authors and amplify all the *Theileria* species and stocks we tried, we felt that with a combination of the two primer sets we would be able to detect a wide range of the *Theileria* parasites that may be present in the areas sampled. It is highly probable that the primers will also amplify species of *Theileria* which have not yet been isolated or characterized in laboratories. This may well be the case in Kitale and Kakamega, particularly with the blood samples that presented the hmw band with the TPR1 primers. It is important to characterise *Theileria* species in a *T. parva* 'pre-vaccination' area because if one of the consequences of vaccine administration was to briefly immunosuppress the animals, it is these pathogens which may become far more of a disease risk.

R. appendiculatus is reported as being able to transmit *T. parva* stocks, *T. taurotragi* and *T. ovis. Rhipicephalus evertsi* can transmit *T. parva, T. ovis* and *T. separata* and *Amblyomma variegatum* can transmit *T. mutans* and *T. velifera.*[9] It is likely therefore, that Limuru will contain only the *Theileria* species that *Rhipicephalus* species can trans-

mit (as *A. variegatum* was not present), while Kitale and Kakamega could contain all of the above mentioned species because of the larger variety of tick vectors in the areas. The infection prevalences in both cattle and ticks varied markedly between the areas.

The difference in the levels of *T. parva* infection detected between the *T. parva* vaccinated and unvaccinated animals in Limuru was small, 40% to 47% respectively. Admittedly, the sample size was relatively small (33 blood samples), but we still felt our results do not support earlier work which reported that application of the ECF vaccine created a carrier state in all of the vaccinated cattle.[10] The PCR-based method we report here have been shown to be sensitive to the level of one piroplasm in 10^7 erythrocytes,[11] which was the same as the sensitivity found for *T. annulata*,[12] but it has not been compared directly with the method used by Kariuki *et al.*,[10] so we are unable to comment on the relative sensitivity of the two tests.

The blood samples collected from Kakamega were all positive by the PCR for *Theileria* species (and piroplasms were seen in all of the blood smears), but *T. parva* was not detected in any of the samples by the PCR. The piroplasms all looked atypical of *T. parva*, but identifying particular species of *Theileria* in blood smears from field infections can be very difficult owing to the low numbers of piroplasms present. Amplification from the *Amblyomma* salivary gland is most likely to have come from *T. mutans* but since we were unable to amplify DNA extracted from a stabilate we have in the laboratory with primers 989/990, described as being *T. mutans* with the primers used, cannot confirm this at the present time.

The PCR results from ticks have to be interpreted even more carefully than the blood results. The favored situation is when only the salivary glands are analyzed, but this is not always possible as dissection requires live ticks. If the ticks were dead, DNA was extracted from a bisected sample, but it must be borne in mind that a 'false positive' can be obtained from parasites present in the blood meal in the gut which can disguise the detection of the presence or absence of parasites within the salivary glands, which is the only stage of relevance to direct transmission. This effect has been noted before in co-feeding infections within ticks with viruses[13] and *T. parva* infections.[14] This problem can largely be overcome however, by bearing in mind the disease situation in the host on which the tick was feeding. For example, a positive result from a blood-fed tick on an uninfected host would indicate a salivary gland infection as would the detection of a different species of *Theileria* within the tick than was present in the bovine blood. Two *Boophilus* samples which gave positive detection of *T. parva* in Limuru had been prepared by pooling 2 and 3 nearly engorged female ticks which were then bisected. The bovine they were feeding on was a carrier animal for *T. parva* (as confirmed by microscopy and the PCR) and the positive result may have come from the large amount of infected blood that was present within the guts of the ticks, as *Boophilus* ticks are not vectors of *T. parva*.

The tick infection level in Limuru was very similar to other reports studying the prevalence of *T. parva* in *R. appendiculatus* field collected ticks[15–17] and to a study carried out by the authors several months previously which involved collecting ticks from a paddock around the NVRC, Muguga, Kenya (data not shown). The infection level in Kitale was substantially higher at 14%, but the small sample size of 94 ticks precludes any serious comparative judgements. Several reasons can be put forward for this, including the 100% cattle infection level in Kitale compared with only 43% in Limuru and the larger variety of tick vectors in the area, especially as they were feeding on exotic cattle breeds as opposed to the less susceptible zebu-borans that are present in Kakamega. Only one *R. appendiculatus* in Kakamega was detected as being *Theileria* positive. Although the *Theileria* like parasites found in the Kakamega area have not been characterized, it may be that they are mainly species which are not transmitted by

Rhipicephalus or *Amblyomma* ticks or that infect only a very small proportion of the ticks, in which case, detection may be reliant on a larger sample size. That all the animals in the area sampled were infected is testament to the fact that infection does occur.

We were unable to detect *T. parva* in cattle and ticks in the area, even with the large number of *R. appendiculatus* present. In a situation like this, it may be prudent to exercise caution in introducing a vaccine into an area where no *T. parva* has been detected, as it may serve to introduce a highly pathogenic species into an area containing a high proportion of tick vectors. This may actually serve to create a long-term need for the vaccine, and as the vaccine would be a commercial enterprise, it would represent a serious economic burden on the farmers in the area.

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Pathological effects and reduced survival in *Rhipicephalus appendiculatus* ticks infected with *Theileria parva* protozoa

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Introduction

There is an increased understanding of arthropod vectors as organisms which are themselves susceptible to the harmful effects of the vertebrate pathogens they transmit (Beier 1998; Zheng 1997). The vectors have the capacity to mount an innate and acquired immune response towards infection (Lowenberger et al. 1996; Richman et al. 1996). However, by definition, if they are biological vectors they must be susceptible to infection, and such infection has a potential to cause harm at a level detectable by conventional pathology and observations on survival. Such is the case with insect vectors. For example, mosquitoes infected with viruses for which they are the natural vector suffer reduced survival (Faran et al. 1987); altered feeding behaviour (Grimstad et al. 1980) and cytopathological effects (Mims 1966). *Boophilus decoloratus* infected with *Babesia bigemina* (Gray, 1982) and *Hyalomma excavatum anatolicum* infected with *Theileria annulata* (Schein & Friedhoff, 1978) both suffer reduced survival as a result of infection with the protozoa. High prevalences of natural infection of *Hyalomma a. anatolicum* have been found to be very intense (Sangwan et al. 1986; Walker et al. 1983) and we find that similar abundances of infection in laboratory ticks causes reduction in size and effectiveness of tick salivary glands (E. Kirvar, personal communication). The epidemiological significance of such phenomena is the effect they could have on the combination of susceptibility to infection with *Theileria* of ticks and the numbers of ticks available to infest cattle. These two parameters combined with susceptibility of the cattle constitute the simplest model of risk of infection to the cattle (Medley et al. 1993). Small variations in infection prevalence and tick survival may have large effects on this risk.

Thus we have searched for evidence of these phenomena in *R. appendiculatus* ticks fed on a calf infected with *T. parva* in the context of studies on vector competence. Our observations on infected ticks are compared with previous anatomical studies in this laboratory on *R. appendiculatus* fed on uninfected rabbits and calves (Walker et al. 1985; Walker & Fletcher 1987) and similar studies on this tick by Fawcett et al. (1981, 1982).

Materials and methods

Calf and *Theileria*:

A male Friesian calf of 9 months of age and 210kg weight, which had no previous exposure to ticks or *Theileria* parasites was used. It was inoculated above the right prescapular lymph node with a sporozoite stabilate of *T. parva* Muguga. The stabilate was prepared from

infected adult *R. appendiculatus*, and the inoculum was equivalent to approximately 150 sporoblasts of *T. parva*. The course of *T. parva* infection was monitored by rectal temperature, palpation of the right prescapular lymph node, biopsy samples from this lymph node and venous blood films. Biopsy material and blood were stained with Giemsa's stain and examined for macroschizonts and piroplasms respectively. Tetracycline antibiotic (Engemycin) was administered daily at 10mg/kg of calf weight for 5 days after the calf's temperature exceeded 40^0C. This was to prolong the period of high piroplasm parasitaemia; use of this antibiotic alone is not associated with harmful effects on the ticks and is regularly used for *T. parva* transmission experiments by other researchers (Ochanda et al. 1996). Calf pyrexia (anal temperature exceeding 39.5^0C) was recorded on day 7 post-infection and this was also the day in which macroschizonts were first detected in the local drainage lymph node to stabilate injection. *T. parva* piroplasms were recorded from day 12 and rose from 0.2% to 22.4% on day 17 at which point the animal was killed. All use of experimental animals was under the sanctions of the Animals (Scientific Procedures) Act 1986 of the United Kingdom.

Ticks:

Rhipicephalus appendiculatus nymphs that had been fed as larvae on rabbits 6 months previously were used. They were applied to the calf on day 10 post-infection in cloth bags attached to the neck and detached from days 14 to 17. Detached, engorged nymphs were collected daily, then maintained at 28^0C, 85% r.h. Control ticks consisted of nymphs from the same batch fed on a rabbit, and from histological material from a previous experiment with *R. appendiculatus* nymphs fed on uninfected calves (Walker & Fletcher 1987). The survival of the infected and uninfected nymphs through moult to adults was counted. The reproductive performance of test and control adult ticks that resulted from the feeds of nymphs was examined by applying them separately to each ear of one rabbit, with replicate feeds on another rabbit. Records were made of percentage females engorged, mass of females at engorgement, mass of eggs at completion of oviposition and percentage of eggs hatching to larvae.

Histology:

Fed nymphs, both infected with *T. parva* and uninfected controls were removed from the maintenance incubator on days 0, 2, 4, 6, 8, 9, 12, 13, 15, 17, 19 and 21 post-detachment and

processed for histological examination. The ticks were attached to a wax dissecting dish, immersed in 1% saline and the dorsal cuticle removed. The ticks' viscera were flushed with Karnovsky's fixative. The whole mass of partially fixed viscera was then removed from the ventral cuticle and transferred to fresh fixative for 2 hours. For light microscopy, the specimens were embedded in hydroxy-ethylmethacrylate (Historesin, Leica Microsystems UK Ltd) using infiltration with the monomer over 4 days, then sectioned at 2μm on an ultramicrotome. The sections were dried onto glass slides, stained with Giemsa's stain and mounted in plastic with a coverslip. The number of individual ticks sectioned and examined on any one sampling day ranged from 7 to 15. In total, sections from 108 ticks were examined. For transmission electron microscopy, only ticks between days 15 to 21 were examined. Post-fixation used osmium tetroxide, embedding was in epoxy resin (Araldite type) with infiltration over 4 days, and staining used Reynold's lead citrate. In addition, ticks that had completed moult by day 28 were dissected to remove all viscera and the internal surfaces were prepared for scanning electron microscopy by critical point drying and coating with gold and palladium. The semi-thin sections were examined to count *T. parva* forms, which were identified using the criteria of Mehlhorn et al. (1978), Mehlhorn & Schein (1984) and Schein et al (1977) and by comparing infected and uninfected ticks.

At completion of post-moult development, adult ticks were dissected and examined for *T. parva* infection. They were incubated at 37^0C, 100% r.h. for 5 days before salivary gland dissection, staining with methyl green and pyronin (Walker et al. 1979) and examination for sporoblasts. Twenty male and twenty female ticks were examined from each nymphal detachment day unless there was an inadequate number of surviving ticks from that day; a total of 112 adult ticks were examined.

Results

Enumeration of *T. parva*

Table 1 shows the number of *T. parva* forms counted on different sampling days throughout the ticks' moult. The number of gametes decreased from days 0 to 2 post-detachment, and no *T. parva* forms were seen on days 4 and 6. Zygotes were first seen in sections of day 8 ticks. The first peak of zygotes and kinetes was seen on day 9 post-detachment, which showed a mean number of 76 zygotes and 2.8 kinetes per section. All zygotes and kinetes were seen only in the gut. Zygote numbers dropped substantially on days 12 and 13 to a mean of 5.6 and

1.1 zygotes per section respectively. The second, smaller peak of *T. parva* zygotes and kinetes, seen on day 15 post-detachment showed a mean of 20 zygotes and 0.4 kinetes per section and by day 17 this had dropped to a mean of 6.3 zygotes and 0.1 kinetes per section. Sporonts were first seen in salivary gland acini on day 17 and sporoblasts on day 19 post-detachment.

Effects on tick survival, the gut and salivary glands:

Up to days 8 or 10 post-detachment, there was little difference in the development of the gut between the uninfected and infected group of ticks (Fig. 1). Gross pathological signs became evident in infected ticks from day 9 onwards in that their moult became slowed in the majority of ticks, and stopped in others compared to the controls. Tick mortality increased from 5 to 100% in batches that detached over the course of the rising calf piroplasm parasitaemia (Table 2). Approximately 20% of the survivors from the batches that detached at the highest parasitaemias were crippled with missing or distorted legs. From day 9 post-detachment onwards, a vacuolated appearance in the basal area of the sessile digestive cells was seen in histological sections of the infected ticks (Fig. 2). This appeared to be caused by a coalescence of lipid vesicles. On dissection of infected ticks it was seen that some areas of the guts were very fragile compared to those of uninfected ticks. This corresponded to the appearance by light microscopy of some gut areas without a structured epithelium and only the basal lamina provided remaining structural integrity (Fig. 3).

Theileria kinetes were seen in the gut sessile digestive cells (Fig. 4). A number of examples showed evidence of ultrastructural, pathological signs in comparison with published electron micrographs of such kinetes (Mehlhorn et al. 1978) (Fig. 5). The outer cell membrane was clearly defined, but no mitochondria or profiles of endoplasmic reticulum were seen. Around the periphery of the kinetes, in the cytosol of the digestive cells a homogenous, structureless layer was seen which contrasted with the normal profiles of endoplasmic reticulum and vesicles.

By day 17, the salivary glands of infected ticks showed pycnotic and metachromatic structures specifically in *e* cells of type 3 acini (Fig. 6). These were green, dense, refractile and granular. Large vacuoles were later seen in the same cells in infected ticks (Fig. 7). Within affected acini there remained normal *e* cells and other cell types (Fig. 8). The *e* cells that contained the pycnotic and metachromatic structures were dominated by this pathological

effect and appeared moribund. By electron microscopy, the affected *e* cells were seen with cytoplasm with a homogenous structure, lacking normal profiles of endoplasmic reticulum seen in adjacent cells (Fig. 9). On dissection, the salivary glands of infected ticks were commonly less well developed than in uninfected ticks. There were bare, branched ducts and acini were only present on the distal ends of the ducts.

Kinetes were not seen penetrating salivary gland acini or within them. However, *T. parva* sporonts were seen by light microscopy by day 19 post-detachment. These were located in *e* cells of type 3 acini as defined by the characteristic secretory granules of these acini, which remained in small numbers in infected cells.

Salivary Gland Infections:

A non-linear relationship was seen between the level of sporoblast infections in the adult ticks and the calf's piroplasm parasitaemia on the day of nymphal detachment. Table 2 shows the prevalence and abundance of infection in the adult tick salivary glands and the batch mortality in relation to the calf parasitaemia upon detachment. The prevalence and abundance values drop from 100 and 129 respectively at the lowest piroplasm parasitaemia to around 27.5 and 12 at the highest parasitaemia, even though the number of piroplasms ingested by the ticks would have been 2 – 7 times greater at the higher parasitaemias.

The effect on feeding and reproduction:

Uninfected, engorged females detached from the rabbits over a 3 day period from days 6 to 8 (after placement on the host). Infected females detached over a 4 day period from days 7 to 10. A two-tailed Mann-Whitney test revealed the infected females detached significantly later than the controls ($P<0.0001$, U-statistic = 472, U' + 1928).

The uninfected females imbibed larger bloodmeals than the infected ticks. The mean weight of uninfected ticks was 436mg compared to 298mg for the infected ticks (Table 3). A two-tailed, unpaired *t* test revealed a significant difference between the means of the two groups ($P<0.0001$, $t = 5.875$, 96 d.f). The egg batch weight of the uninfected ticks was 281mg compared to 185mg for the infected ticks. A two-tailed, unpaired *t* test revealed the mass of the control ticks' egg batches were significantly greater than that of infected ticks ($P<0.0001$, $t = 6.405$, 96 d.f). The mean engorged tick weight / egg batch weight ratio for uninfected ticks was 64% compared to 62% for infected ticks.

There was a large difference between the numbers of hatched larvae. The uninfected ticks' egg batches had a mean hatch rate of 61% compared to 12% for the infected ticks' egg batches. A two-tailed, Mann Whitney test revealed a significant difference between the medians of the two groups ($P<0.0001$, U statistic = 363, U' = 1940).

Discussion

The gut of infected and uninfected ticks looked very similar up to and including day 8 post-detachment. Gametes were seen in the gut lumen up to day 2, but no parasites were seen in the gut again until day 8 when zygotes were detected in the cytoplasm of digestive cells. The number of individual *Theileria* parasites do not increase in their tick vectors until they undergo sporogony in the salivary gland acini. Therefore, the large increase in zygote numbers recorded on day 9 (Table 1) is likely to reflect our ability to detect the newly enlarged parasites as opposed to an actual increase in parasite numbers. The first notable tick pathology occurred at this time. The gut became highly vacuolated and showed close similarity with pathology observed in the gut of the reduviid bug *Triatoma infestans* infected with the trypanosomatid *Blastocrithidia triatomae* (Schaub & Neukirchen 1992). At a macroscopic level, the moult of infected ticks was clearly inhibited compared to the uninfected ticks. These effects may have resulted from the stress caused by *Theileria* growth in the gut at this time.

It was originally intended to use a single batch of ticks from one nymphal detachment day throughout the entire processing series for the infected ticks. The number of engorged nymphs collected from the calf on the day it displayed a piroplasm parasitaemia of 19.6 – 22.4% was large enough to allow this. These nymphs were processed and examined only up to day 12 after which their use was discontinued because of irregularities in their moulting performance. The number of *Theileria* forms seen in some of the sections from these ticks was extremely large (Table 1), and the number of zygotes and kinetes peaked on day 9 post-detachment. This was considerably earlier than in batches of nymph / adult ticks examined from other nymphal detachment days in the experiment described here and in *T. parva* infected ticks described in previous reports (Schein et al. 1977; Young & Leitch 1980). It is thought therefore, that the first peak of zygotes and kinetes shown in Table 1 represented an artefact of an abnormal relationship between the parasite and tick batch from that particular day of nymphal detachment. The parasite population within the ticks may have been greater

than the ticks' ability to support them, leading to considerable pathology and 100% mortality seen within that group. *Plasmodium yoelli* induced pathology in *Anopheles stephensi* mosquitoes was reported by Maier et al. (1987). Electron micrograph images showed damaged epithelial cells of *A. stephensi* infected with *P. yoelli* that were clearly protruding from the regular epithelial layer. They commented that mosquitoes could tolerate a certain amount of damage to the epithelia, as neighbouring cells or cellular regeneration replaced tissue, but, if the lesions became too numerous, tissue repair was not possible and the mosquitoes died.

The number of *T. parva* zygotes and kinetes decreased markedly after they reached a peak in numbers in the gut digestive cells. The reduction in the number of these gut forms was not matched by an equivalent increase in the number of sporonts or sporoblasts in the tick salivary glands. We have little evidence on mechanisms of immune reduction of *Theileria* infections in these ticks, but suggest that intracellular destruction of zygotes and kinetes in the gut digestive cells may occur. A similar mechanism has been described in *Anopheles gambiae* mosquitoes that are refractory to *Plasmodium gallinaceum* infection. The precise killing mechanisms were not elucidated, but ookinetes were destroyed in the gut epithelial cell cytoplasm without melanotic encapsulation occurring (Vernick et al. 1995).

The pathology that appeared in the salivary glands by day 17 followed the appearance of kinetes in the gut digestive cells at day 15. As sporonts were also seen in the salivary gland acini by this time, kinete penetration must have occurred during this period. No kinetes were seen in the haemolymph in any tick sections examined, and no examples were noted of kinetes in the process of acinar penetration.

The prevalence and abundance of *T. parva* infection in the adult tick salivary glands showed a non-linear relationship to the level of piroplasm parasitaemia in the calf at the times of nymphal detachment. The reduction in these values, derived over the course of nymphal detachment would have been considerably more marked if data from the total number of sporoblasts within surviving adult ticks, divided by the total number of ticks that detached from the calf on any one day had been used in the calculation. The figures calculated from the ticks that detached at higher piroplasm parasitaemias were based on the survivors of populations that suffered extremely high mortality as a direct result of *T. parva* infection. The reasons for reduced tick infections as a consequence of higher piroplasm ingestion are unknown.

T. parva infected *R. appendiculatus* females showed significantly different feeding and reproductive behaviour than uninfected controls. The pathological effects on the gut and salivary glands are likely to impair infected female ticks' ability to feed, which would account for the lower engorgement weights relative to controls. However, the percentage conversion of body mass to egg batch mass was the same in infected and control ticks. This suggests that the ovaries had not been affected by the pathology evident in other organs.

The range data displayed in Table 1 clearly shows there is large variation in the number of *T. parva* forms seen throughout every stage of the tick moult. An overdispersed distribution of *Theileria* sporoblasts is usually encountered in laboratory infected ticks (Büscher & Otim 1986). The data presented here suggests this variation is present in all tick forms of *T. parva*. Considering that most ticks collected from a single day's detachment will have ingested a similar number of piroplasms, it is possible that individual variations in tick immune responses may be among the factors affecting the apparent variation in parasite numbers.

The pathology and consequent mortality in the tick batches described here is unusual in the extent to which it occurred. We have examined histological sections from a number of other *T. parva* infected tick batches, which did not show any obvious abnormalities as a result of infection (data not shown). We observed similar types of pathology, particularly in the salivary glands, but on a much smaller scale. It is likely that a wide spectrum of *T. parva* induced, pathological effects in *R. appendiculatus* occurs and that this paper describes an extreme example of this. However, these effects should be considered in the formulation of epidemiological models of theileriosis.

There are a number of unanswered questions that arise from this work. The reasons why such severe pathology and mortality should have resulted in batches of ticks that detached on specific days are not known, but would merit further investigation. A number of factors may be involved such as the age and turnover of piroplasms on the day preceding engorgement (Purnell et al. 1974) and clinical manifestations in the calf on that particular day.

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Table 1. The numbers of *T. parva* forms were counted in Giemsa stained, histological sections of *R. appendiculatus* throughout the period of the ticks' moult. The table shows the results of these counts from newly detached nymphs at day 0 (post-detachment) through to fully developed adults at day 22.

Day	Piroplasm %	n	Mean No. of Gametes	Mean No. of Zygotes	Mean No. of Kinetes	Mean No. of Sporonts	Mean No. of Sporoblasts
0	19.6 - 22.4*	9	168 (16 - 415)				
2	19.6 - 22.4*	13	18 (1 - 71)				
8	19.6 - 22.4*	13		0.7 (0 - 4)			
9	19.6 - 22.4*	10		76 (3 - 217)	2.8 (0 - 13)		
12	19.6 - 22.4*	9		5.6 (3 - 10)			
13	22.4 - 22.4	15		1.1 (0 - 10)			
15	13.1 - 19.6	14		20 (0 - 66)	0.4 (0 - 3)		
17	13.1 - 19.6	10		6.3 (0 - 50)	0.1 (0 - 1)	1.3 (0 - 7)	
19	13.1 - 19.6	7		0.3 (0 - 1)		3.0 (0 - 11)	0.1 (0 - 1)
21	13.1 - 19.6	12		0.1 (0 - 1)		0.3 (0 - 2)	0.4 (0 - 2)

Piroplasm % is the piroplasm parasitaemia on the day of tick detachment, n is the number of ticks sectioned and examined by light microscopy.
* - these ticks showed abnormalities in their moulting behaviour by day 13 and all died by day 17. The figures in brackets represent the range in numbers of *T. parva* forms counted.

Table 2. Variation in tick batch mortality and the prevalence and abundance of *Theileria parva* sporoblasts in *Rhipicephalus appendiculatus* adult salivary glands in relation to the calf piroplasm parasitaemia on the day of tick detachment.

Day Post Infection*	Piroplasm Parasitaemia (%)	n†	Sporoblast Prevalence (%)	Sporoblast Abundance‡	Tick Mortality (%)
14	3.2 – 13.1	40	m: 100 / f: 100	m: 129 / f: 129	5
15	13.1 – 19.6	40	m: 70 / f: 70	m: 29 / f: 88	30
16	19.6 – 22.4	n.a.	n.a.	n.a.	100
17	22.4 – 22.4	28	m: 29 / f: 26	m: 11 / f: 13	75

Notes: m - male ticks, f - female ticks, n.a. – not applicable because of complete batch mortality, * - refers to the time after calf infection. † - number of ticks processed, ‡ - abundance refers to the number of *T. parva* sporoblasts counted, divided by the total number of ticks examined in that batch.

Table 3. Comparison of the survival through moult and reproductive performance of *Rhipicephalus appendiculatus* fed as nymphs either on a calf infected with *Theileria parva* (infected ♀), or on rabbits (control ♀), and then both groups fed as adults on rabbits.

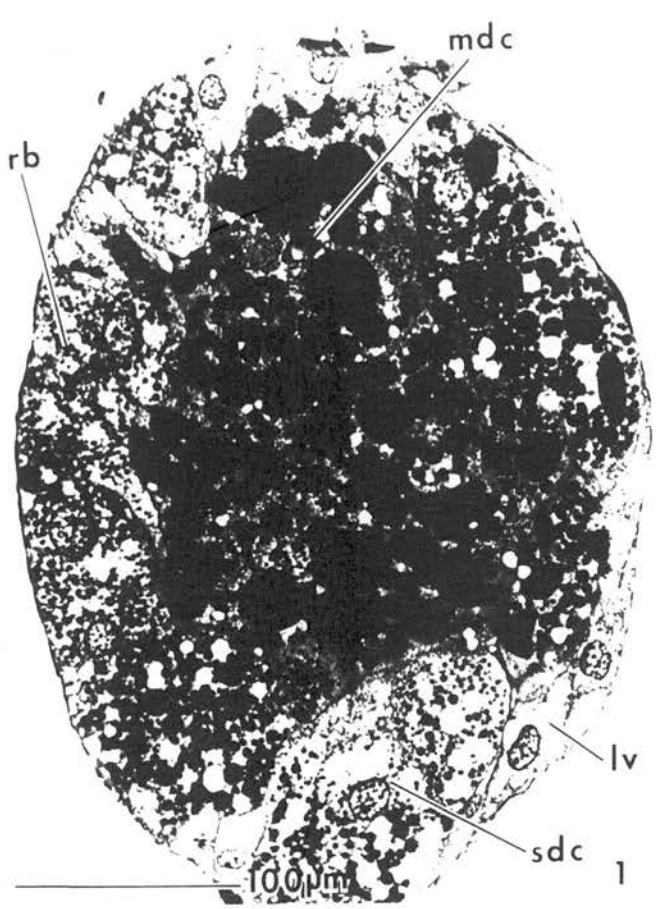
Criterion	Infected ♀	Control ♀
Mean % Moult	0 - 95	99
Engorged (%)	100	100
Mean Mass (mg)	298	436
Mean Egg Batch Mass (mg)	185	281
Mean % Eclosion	11	62

Notes: The infected ticks were fed as nymphs on calf 48A infected with *T. parva* and then fed as adults on one ear each of two rabbits. The control ticks were fed as nymphs on a separate rabbit and then as adults on the opposite ears of the two rabbits used for the infected ticks. The variation in the percentage of test nymphs moulting to adults depended on the day of detachment.

Figures

Figs 1-5. (1) Light micrograph of gut section of uninfected *R. appendiculatus* at day 19 post-detachment (p.d), showing accumulation of residual bodies (rb) in sessile digestive cells (sdc), discrete lipid vesicles (lv) and uniform composition of motile digestive cells (mdc). (2) Light micrograph of gut section of infected *R. appendiculatus* at day 18 p.d showing in comparison to Fig. 1 fewer residual bodies, coalesced lipid vesicles, and heterogenous composition of motile digestive cells. (3) Light micrograph of gut section of infected *R. appendiculatus* at day 15 p.d showing disintegrated epithelium with protein vesicles (pv) and the basal lamina (bl). (4) Light micrograph of gut section of infected *R. appendiculatus* at day 15 p.d showing kinete (ki) of *T .parva* characterised by vermicule shape, within a sessile digestive cell. (5) Electron micrograph of infected *R. appendiculatus* at day 15 p.d showing a kinete of *T. parva* with peripheral micronemes (mi), poor differentiation of central cytoplasm (cy) and a surrounding homogenous layer (arrowed) in cytoplasm of the sessile digestive cell compared to the more heterogenous structure of the cytoplasm (arrowed) elsewhere in the host cell.

Figs 6 - 9. (6) Light micrograph of section of salivary glands of infected *R. appendiculatus* at day 15 p.d showing retarded development and pycnotic and metachromatic structures (arrowed) in a type III acinus; other uninfected type III acini (ac3) are shown. (7) Light micrograph of infected *R. appendiculatus* at day 17 p.d showing pycnotic and metachromatic structure (arrowed) in a type III acinus; two unaffected type III acini (ac3) are shown and the remaining acini are type II. (8) Electron micrograph of a type III acinus from an infected tick at day 17 p.d; the left hand *e* cell (ec) shows a pycnotic structure of coalesced secretory granules (csg); the central *e* cell has degraded cytoplasmic structure and the right hand *e* cell is normal; the upper three cells are *f* cells (fc) which are agranular at this stage of development. (9) An area of Fig. 8 to show the degraded homogenous cytoplasm of the upper *e* cell compared to the normal cytoplasm of the lower *e* cell; the secretory granules (sg) are normal in both cells, the position of this area is arrowed in Fig. 8.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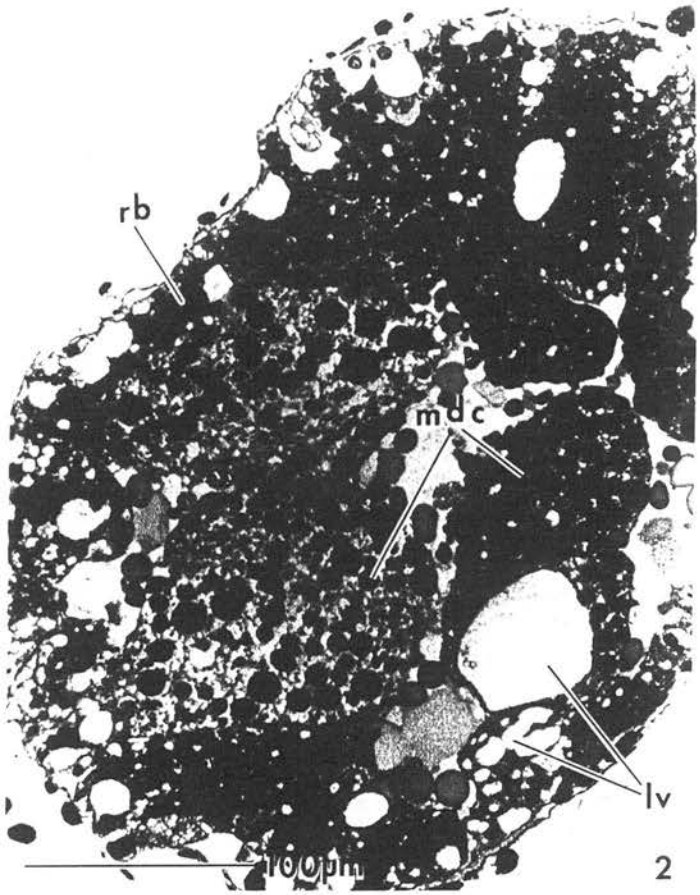
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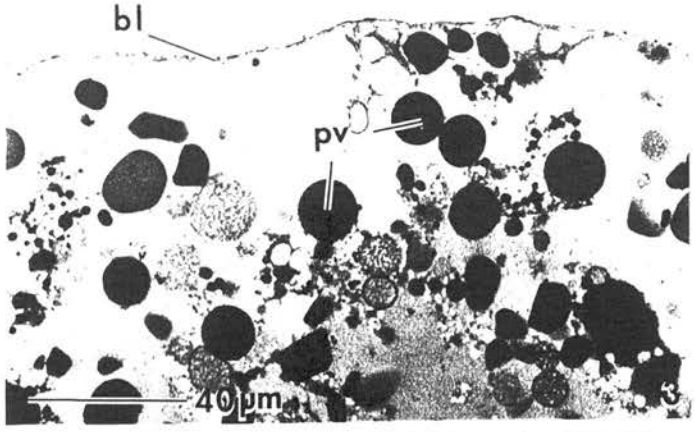
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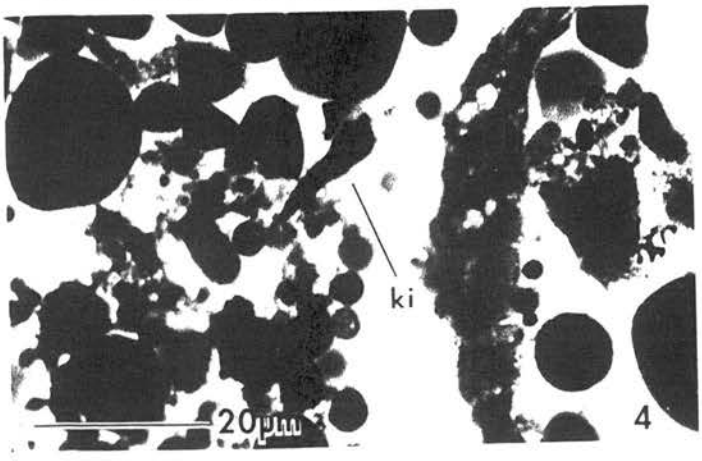
3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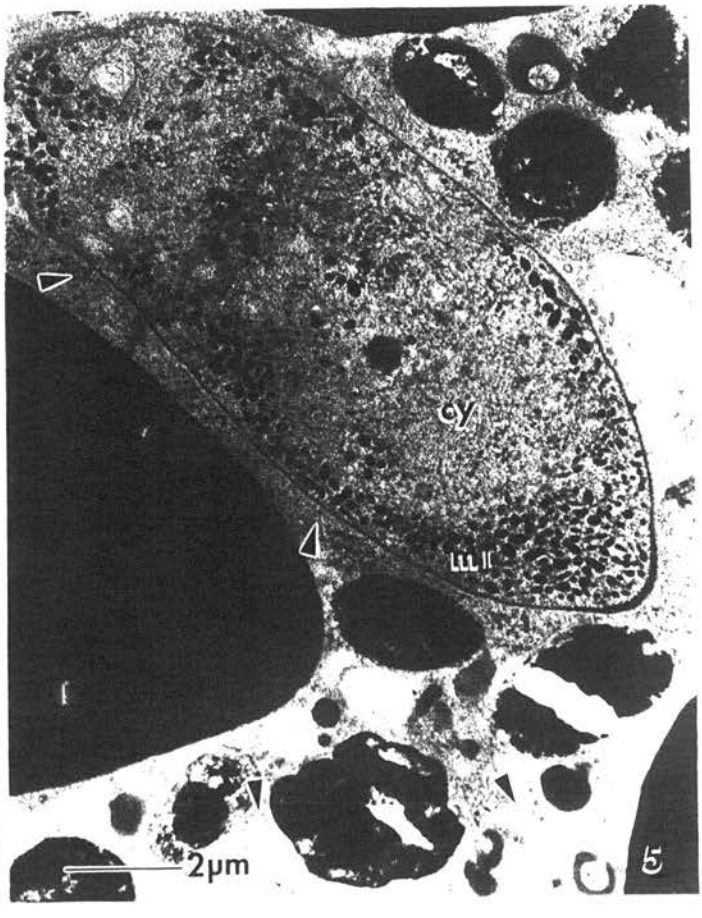
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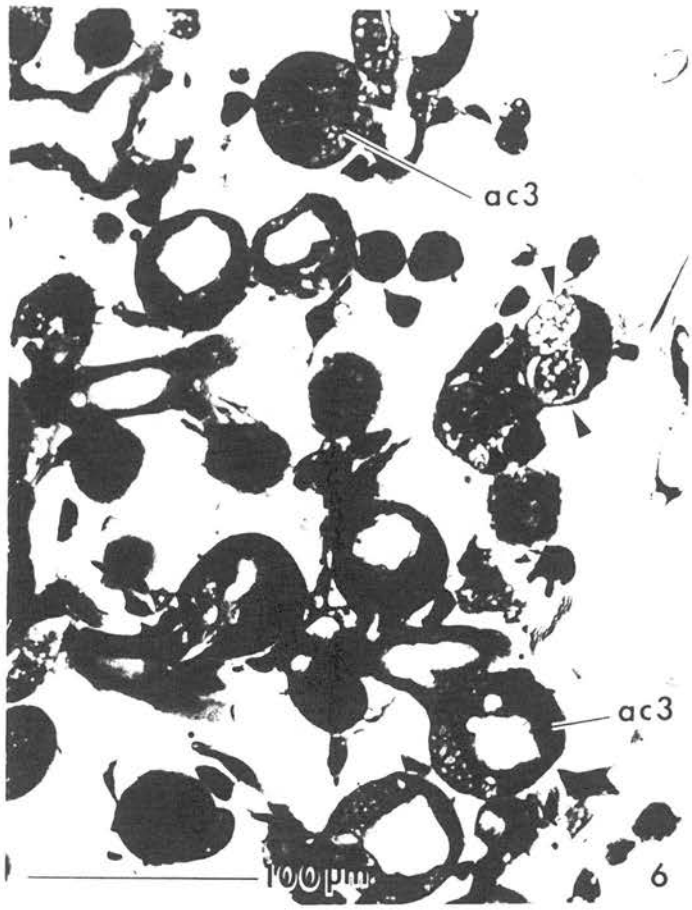
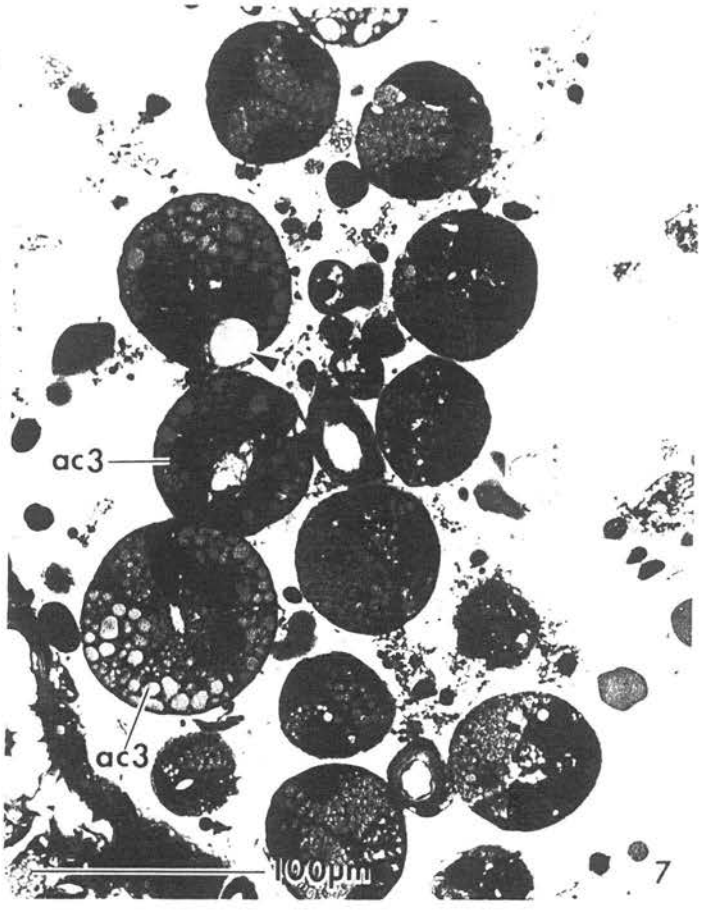
2µm
5


ac3
ac3
100µm
6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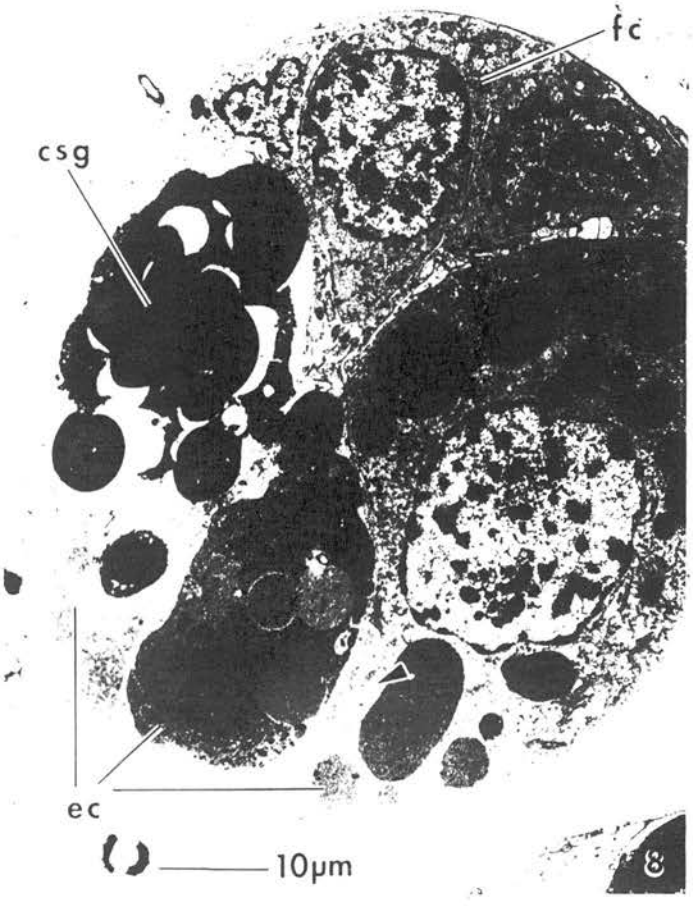
7


fc
csg
ec
10µm
8


sg
sg
1µm
9